ABSTRACT

CO-EXPRESSION OF REGULATOR OF G PROTEIN SIGNALLING 4 (RGS4) AND THE MU OPIOID RECEPTOR IN REGIONS OF RAT BRAIN: EVIDENCE THAT RGS4 ATTENUATES MU OPIOID RECEPTOR SIGNALLING

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Directed by Thomas E. Cote, Ph.D., Associate Professor of Pharmacology and Neuroscience

Regulators of G protein Signalling (RGS) proteins influence G protein-coupled receptor signal transduction by enhancing the intrinsic GTPase activity of G proteins. The RGS-enhanced GTPase activity of G proteins may be responsible for the desensitization of certain G protein-coupled receptors, including the mu opioid receptor. The goal of this research was to evaluate the ability of recombinant RGS4 to affect mu opioid receptor-mediated cellular signalling and to identify regions of the rat brain in which both RGS4 and the mu opioid receptor are co-expressed.

We evaluated the ability of recombinant RGS4 to affect [D-Ala^2, N-Me-Phe^4, gly-ol] enkephalin (DAMGO)-mediated inhibition of adenylyl cyclase activity in membranes of SH-SY5Y cells, a cell line that express endogenous mu receptors.

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14. ABSTRACT

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Together, these findings are consistent with the proposal that RGS4 can desensitize mu opioid receptor by increasing the intrinsic GTPase of Gi-type G proteins associated with the mu opioid receptor and that, *in vivo*, RGS4 and the mu opioid receptor are co-expressed in many of the same regions of the rat brain.

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by

A. Tamara Crowder

Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2003.

DEDICATIONS

To Eddie and James

In memory of Tony

And for Martha, I am truly the most fortunate person on earth to have her as my friend.

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The successful completion of a doctoral dissertation reflects the combined efforts and influences of the many people who help to shape the student's life. I am especially grateful to my dissertation committee for their tireless devotion to science. I consider myself to be extremely fortunate to have had so many individuals commit to the development of me as a scientist. My advisor, Thomas Cote, has been a wonderful mentor, colleague, and friend. He spent years guiding my growth as a scientist, teaching me to write effectively and to critically evaluate the literature, to develop a long-term research plan followed by methodical, appropriate experimental design. He also taught me more in more subtle ways, giving me the freedom to work independently, to try some things outside the box. He has been a dear friend and an integral part of my life. His patience is remarkable, whether editing one of my countless grant proposal drafts, or teaching my son to throw a slider. Working in his laboratory has been an intellectually stimulating, wonderful experience, both on a professional and personal level.

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LIST OF ABBREVIATIONS

ANP	atrial ntriuretic peptide
B_{max}	concentration of drug that results in maximal receptor occupancy
cAMP	adenosine 3' 5' –cyclic monophosphate
cGMP	guanosine 3' 5' –cyclic monophosphate
DAMGO	[D-Ala^2, N-Me-Phe^4, gly-ol] enkephalin
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
GAPs	guanosine triphophatase-activating proteins
GDP	guanosine 5' diphosphate
Gi	inhibitory G protein heterotrimer that hyperpolarizes the cell by increasing K^+ conductance
Go	inhibitory G protein heterotrimer that decreases Ca^{2+} conductance
GPCR	G protein-coupled receptor
GRK	G protein receptor kinase
GST	glutathione-S-transferase
GTP	guanosine 5' triphophate
GTPase	guanosine 5' triphophatase
IPTG	isopropyl □-D-thiogalactaside
kDa	kilodalton
K_D	concentration of free drug that results in half-maximal binding

MAPK	mitogen activated protein kinase
MOR	mu opioid receptor
nm	nanometer
PBS	phosphate buffered saline
PDE	phophodiesterase
PKA	cAMP-dependent protein kinase A
PKC	Protein Kinase C
PKG	cGMP-dependent protein kinase
RGS	Regulator of G protein Signalling
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBST	Tris buffered saline + Tween-20
TRITC	tetramethylrhodamine isothiocyanate
U	NIH unit equivalent to the amount of thrombin protease required to cleave ≥ 500 µg of a test GST-fusion protein in 1X PBS at 22C for 16 hour

Chapter 1

Introduction

Format

This dissertation is presented in the alternative format. The introduction is followed by two chapters consisting of manuscripts submitted to peer reviewed journals, a discussion of the research, its significance, future directions, and an addendum consisting of original specific aims which yielded negative results.

Background/Significance

For thousands of years morphine and related opiate drugs have been known as the most effective drugs for the relief of severe pain. Opiates exert their effects by activating opioid receptors, G-protein coupled receptors that are the targets of endogenous opioids and exogenously administered opioid drugs. The clinical usefulness of morphine has been limited, however, because of its ability to induce adaptive changes in brain function, such as tolerance and dependence, following repeated administration (Nestler et al., 1993). Tolerance refers to a progressive decrease in potency and efficacy of the opiate drug that results in diminished analgesia, requiring increasing concentrations over a period of time to achieve the desired analgesic effect. Dependence refers to the need for continued drug administration to avoid withdrawal syndrome, a condition characterized by physical and psychological disturbances following removal of

the drug (Nestler, 1993, 1997). Both tolerance and dependence are mediated by a set of prolonged, activation-induced changes at the receptor and at downstream signalling effectors (Nestler, 1997; Nestler and Aghajanian, 1997; Keith et al., 1998). Because morphine remains one of the most potent analgesics, a better understanding of the molecular mechanisms involved in the development of tolerance and dependence may lead to better strategies for relieving severe pain without causing adverse consequences.

Types of opioid receptors

Morphine and endogenous opioids exert their pharmacological and physiological effects by binding to opioid receptors. Opioid receptors were first identified and characterized in the brain and nervous system using radiolabelled ligands (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). The existence of the three major types of opioid receptors, mu, delta and kappa, were proposed in 1976 based on the pharmacological actions of various opiates in the chronic spinal dog (Gilbert and Martin, 1976; Martin et al., 1976). As a first step toward isolating each receptor type, a number of laboratories solubilized active opioid receptors from rat brain (Chow and Zukin, 1983; Georgoussi et al., 1995; Weems et al., 1996), guinea pig brain (Itzhak et al., 1984), bovine brain (Standifer et al., 1991), and cell lines (Keren et al., 1988; Cote et al., 1993). These studies provided evidence that the three opioid receptor sub-types were separate, well-defined entities with different molecular weights, but all were coupled to pertussis toxin-sensitive G proteins.

All three types of opioid receptors, mu, delta and kappa, were cloned in the early 1990's (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Fukuda et al., 1993; Li et al., 1993; Meng et al., 1993; Minami et al., 1993; Thompson et al., 1993; Wang et al., 1993). Molecular cloning experiments made it possible to study the pharmacological characteristic and molecular mechanisms of receptor function (Raynor K, 1994). The opioid receptors belong to the family of G-protein coupled receptors, each having seven transmembrane regions and sharing a high degree of homology especially in the transmembrane domains and intracellular loops (see Figure 1). Of the opioid receptors, the mu opioid receptor has the highest affinity for medically relevant exogenous opioids, including morphine and codeine as well as for the endogenous opioid peptides. Mice lacking the mu receptor gene lose morphine-induced analgesia, reward effect, and dependence and withdrawal symptoms (Matthes et al., 1996; Sora et al., 1997). These reports indicate that the mu opioid receptor is the major target of morphine and is an essential component in the signalling mechanisms of the pain pathway.

Mechanism of action of the mu opioid receptor

The activation of all three opioid receptors results in the inhibition of adenylyl cyclase activity (Childers, 1991). Collier and Roy (1976) first demonstrated that morphine-like drugs could inhibit adenylyl cyclase in rat brain homogenates. Later, Frey and Kebabian (1984) reported that mu opioid receptor-mediated inhibition of adenylyl cyclase activity in homogenates of 7315c

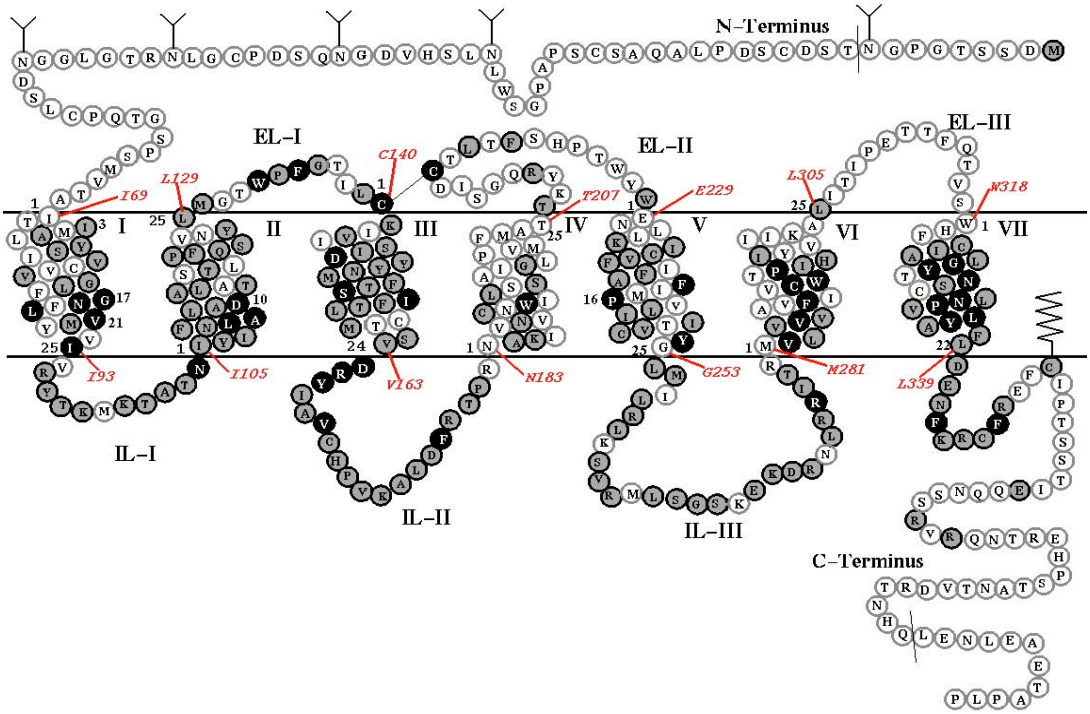

Figure 1. Schematic of mu opioid receptor with its extracellular N-terminus, seven transmembrane domains forming three intracellular loops, and intracellular C-terminal tail. Black residues are homologous to all three opioid receptors, grey residues shared by the mu receptor and either the delta or kappa receptor, and white residues are unique to the mu receptor. C-terminal serine and threonine residues are putative GRK2 phosphorylation sites. Taken from the Center for Opioid Research and Development website with permission of Philip Portoghese.

tumor cells required GTP (Frey and Kebabian, 1984). Further studies demonstrated that the activation of mu opioid receptors in the human neuroblastoma cell line, SH-SY5Y, also resulted in inhibition of adenylyl cyclase activity (Yu et al., 1986). Mu opioid receptor-mediated inhibition of adenylyl cyclase activity was found to be sensitive to pertussis toxin, suggesting the involvement of Gi- and/or Go-type G proteins (Aub et al., 1986; Duman et al., 1988; Fedynyshyn and Lee, 1989; Gosse et al., 1989). Chen *et al*. (1993) established that the cloned mu opioid receptor exhibited the structural features of guanine nucleotide binding protein-coupled receptors and was coupled to adenylyl cyclase via pertussis toxin-sensitive G-proteins.

In addition to inhibition of adenylate cyclase activity, activation of mu opioid receptors also causes hyperpolarization of neurons by increasing K^+ conductance via inwardly rectifying K^+ channels in the locus coeruleus, hippocampus, and submucosal plexus (North et al., 1987; Wimpey and Chavkin, 1991). These mu agonist-mediated increases in K^+ conductance were shown to be sensitive to pertussis toxin, indicating that this effect is mediated by Gi/Go-type G proteins (Tatsumi et al., 1990). Later it was demonstrated that the cloned mu receptor, expressed in *Xenopus* oocytes, could also increase K^+ conductance through activation of pertussis toxin-sensitive G proteins (Chen and Yu, 1994).

Mu opioid receptors also mediate a reduction in neuronal excitability and neurotransmitter release by inhibiting voltage-dependent Ca^{2+} channels (North, 1993). Like many other mu receptor-mediated actions, inhibition of Ca^{2+} conductance is sensitive to pertussis toxin (Suprenant et al., 1990; Seward et al.,

1991). The addition of purified Go to membranes previously treated with pertussis toxin reinstates mu opioid receptor-mediated inhibition of Ca^{2+} conductance (Hescheler et al., 1987). These effects associated with mu opioid receptor stimulation are brought about via the activation of pertussis-toxin sensitive inhibitory G proteins, Gi1, Gi2, Gi3 and Go (Laugwitz et al., 1993; Chakrabarti et al., 1995; Georgoussi Z, 1997). Consistent with these studies, our laboratory demonstrated that Gi1, Gi3, and Go, but not Gs or Gq, co-immunoprecipitate with endogenous mu opioid receptors from rat brain membranes (Chalecka-Franaszek et al., 2000).

Mu opioid receptor agonists also activate Mitogen Activated Protein Kinase (MAP kinase) (Fukuda et al., 1996; Li and Chang, 1996; Polakiewicz et al., 1998). Other laboratories have reported that opioid agonists stimulate phospholipase C□, however neither MAP kinase nor phospholipase C□ has been shown to play a major role in mediating the analgesic effects of mu opioid agonists (Ikeda et al., 2002).(Aub et al., 1986; Yu and Sadee, 1986). Interestingly, inhibition of protein kinase C (PKC) suppressed mu receptor-mediated activation of MAPK in one study and induced an uncharacteristic internalization of the mu receptor in morphine treated cells in another (Fukuda et al., 1996; Ueda H, 2001).

Thus, the mu opioid receptor exerts an inhibitory influence on neuronal activity by inhibition of neurotransmitter release via inhibition of adenylyl cyclase activity and closure of voltage-dependent Ca^{2+} channels and decreases neuronal

excitability by activation of K^+ channels. Each of these signalling pathways may play a role in mu opioid receptor-mediated pain relief.

Desensitization of the mu opioid receptor

Chronic stimulation of mu opioid receptors leads to the development of tolerance. However, the biochemical mechanism underlying the development of tolerance remains unresolved (Nestler, 1993; Borgland, 2001; Taylor and Fleming, 2001). Receptor desensitization, the diminished response of the cell to further mu opioid receptor activation, is one of the mechanisms that may play a role in the development of tolerance (see Figure 2). Perhaps one of the best-characterized mechanisms for receptor desensitization involves agonist-induced phosphorylation of the receptor at serine and threonine residues on the third intracellular loop and the carboxyl terminus of the mu opioid receptor. Several kinases, such as G protein-coupled receptor kinases (GRKs), cAMP-dependent kinase (PKA), and PKC play a role in phosphorylation of these sites. GRK-mediated receptor phosphorylation results in the removal of the receptor from the cell surface by endocytosis. The endocytosed receptor is either dephosphorylated and recycled back to the cell surface or degraded (Zhang et al., 1997; Gagnon et al., 1998; Claing et al., 2002).

G protein-coupled Receptor Kinase 2 (GRK2) has been proposed to play a role in the desensitization of mu receptor signalling following chronic activation of the mu receptor by full agonists, such as DAMGO, fentanyl, or etorphine (Lefkowitz, 1998; Zhang et al., 1998). GRK2-mediated phosphorylation of the

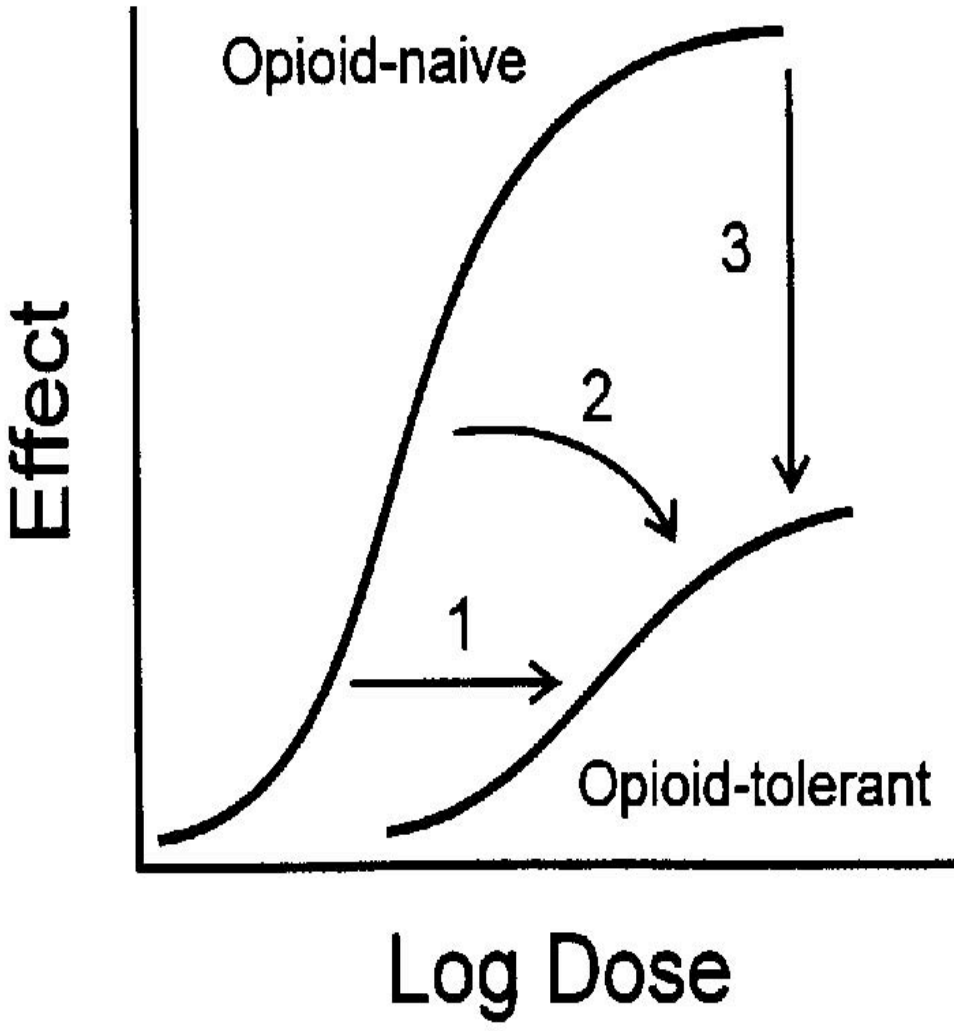

Figure 2. Schematic representation of the development of tolerance. In opioid naive subjects, Increasing effect is attained with increasing dose. In opioid tolerant subjects, effect is blunted, even with maximal dose.

receptor requires that the receptor be occupied by an agonist (Benovic et al., 1986). Phosphorylation of the receptor by GRK2 does not interfere with receptor signalling, but does increase the binding affinity of the protein β-arrestin 2 for the receptor. The binding of β-arrestin2 to the mu opioid receptor interferes with the ability of the receptor to interact with G proteins (Gurevich et al., 1995). This uncoupling effect of GRK2 and β-arrestin2 occurs within minutes of receptor activation. Once β-arrestin2 binds to a receptor, a number of other proteins bind to the β-arrestin/mu receptor complex inducing internalization. The protein dynamin is believed to be the key regulator of vesicle budding because it catalyzes the fission of clathrin-coated vesicles from the plasma membrane (Claing et al., 2002). The internalization process represents a desensitization process because receptors are removed from the membrane, and the internalization may lead to degradation of the receptor. However, the internalization process also is a resensitization process because some internalized receptors are dephosphorylated and returned to the cell membrane (Krueger et al., 1997).

Mu opioid receptor agonists differ greatly in their abilities to stimulate phosphorylation of the mu receptor and to cause internalization of the mu receptor. In HEK 293 cells transfected with mu opioid receptor, the potent opioid agonist etorphine caused a robust GRK2-dependent phosphorylation of the mu opioid receptor. In contrast, morphine failed to cause any detectable phosphorylation of the mu receptor (Zhang et al., 1998). Also, etorphine, but not morphine, caused β-arrestin2 to translocate to the mu receptor and to induce mu

opioid receptor internalization. *In vivo* studies have confirmed that even though etorphine and morphine produced significant tolerance in mice, only etorphine caused a down regulation of the mu receptor density (30% decrease) in spinal cord (Patel et al., 2002). In addition, this work demonstrated that etorphine, but not morphine, caused a 70% increase in levels of dynamin, the protein that plays a key role in mu receptor internalization. These studies suggest that morphine does not cause tolerance via the activation of GRK2 and association of β-arrestin 2 with the mu receptor. However, deletion of the beta-arrestin 2 (βarr2) gene in mice resulted in a prolongation of the morphine-mediated analgesia, and mice lacking β-arrestin2 ($\beta arr^{2-/-}$ mice) failed to become tolerant to morphine (Bohn et al., 1999; Bohn et al., 2000). Therefore, while it is clear that morphine does not cause GRK2/β-arrestin2-mediated internalization of the mu opioid receptor, it appears that β-arrestin2 may play a role in the morphine-induced tolerance. Although the development of morphine tolerance requires chronic receptor activation, it does not result in the adaptational changes at the receptor level, namely internalization and desensitization. Thus, even though a small amount of β-arrestin may associate with the mu receptor, it may be insufficient to cause internalization. However, it may be sufficient to attract other proteins to the mu receptor-β-arrestin 2 complex. One recently discovered class of proteins, Regulators of G protein Signalling (RGS proteins), may become associated with the mu receptor/β-arrestin complex following morphine stimulation to bring about desensitization. The RGS family of proteins works by attenuating G protein-coupled receptor signalling. Signalling is attenuated, or shut off, by binding to the

G subunits through a conserved RGS domain and accelerating GTPase hydrolysis and inactivation (Neubig and Siderovski, 2002).

How RGS proteins work

RGS proteins decrease the period of time that G proteins are active (see Figure 3). Agonist binding to G protein-coupled receptors (GPCRs) prompts the release of GDP from G protein subunits, allowing GTP to bind and activate the G protein. The activated G protein then activates, or inhibits, downstream enzymes or ion channels. Over a period of time, the GTP is hydrolyzed to GDP by a GTPase inherent in the G protein subunit. RGS proteins inhibit G protein-coupled receptor signalling by accelerating the GTPase activity of the subunit (De Vries et al., 2000; Ross and Wilkie, 2000; Neubig and Siderovski, 2002).

Discovery of Regulators of G protein Signalling (RGS) proteins

Regulators of G protein Signalling (RGS proteins) were discovered by Dohlman in *Saccharomyces cerevisiae* (Dohlman et al., 1995). Pheromones in yeast, like most hormones and neurotransmitters in mammals, activate cell surface receptors that are coupled to heterotrimeric G proteins. When a pheromone activates its receptor, the G protein subunit releases GDP, binds GTP, and dissociates from the G protein complex. In yeast, the G complex activates downstream effectors. The G protein subunits remain dissociated and active until GTP is hydrolyzed to GDP, whereupon the subunits re-associate. Dohlman *et al.* (1995) discovered the protein Sst2p (**S**uper-**s**ensitivity **t**o

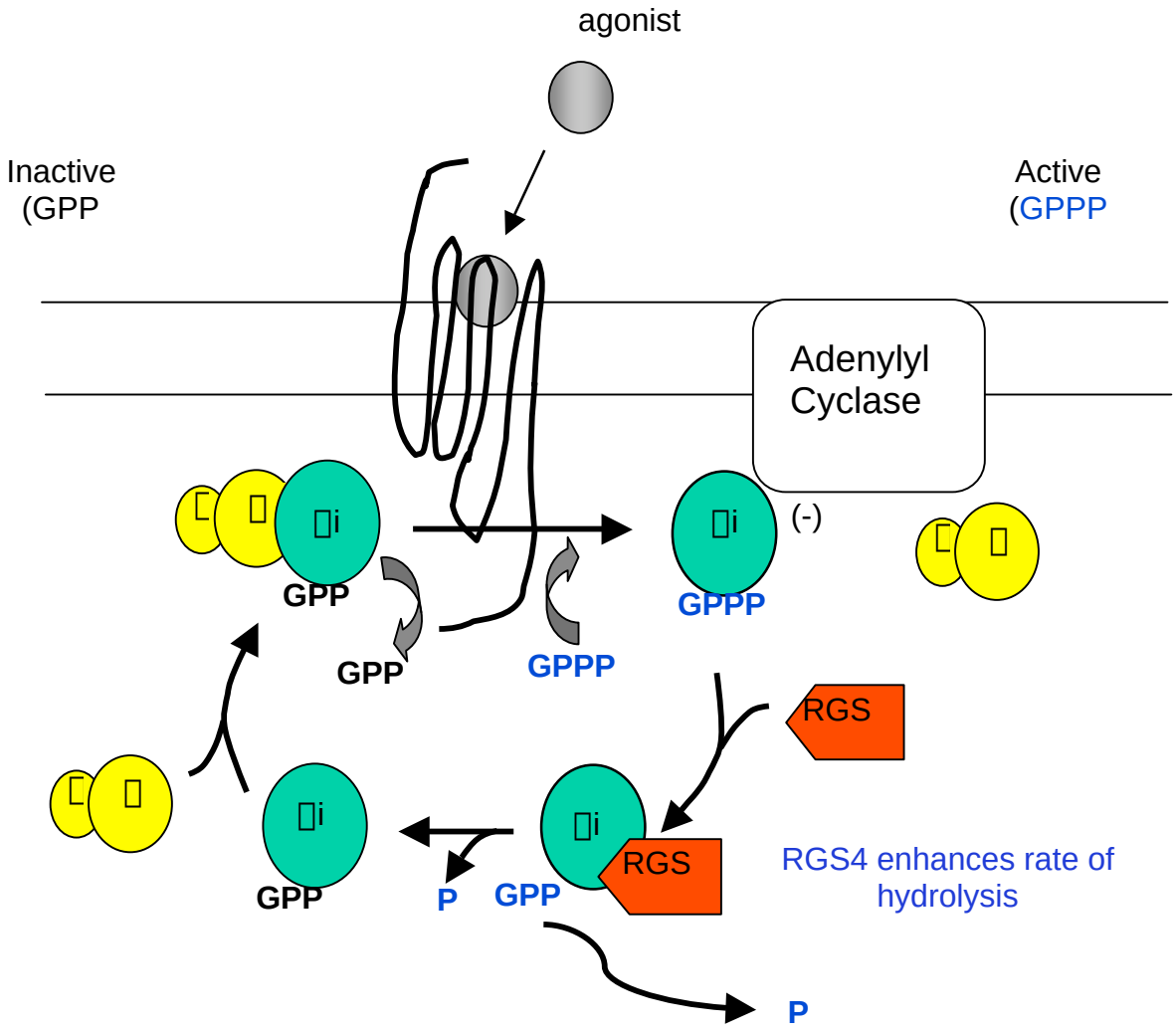

Figure 3. Schematic of RGS function. The binding of an agonist to its receptor causes GDP to be released from the subunit (i) of its G protein (i). The release of GDP (GPP) allows GTP (GPPP) to bind to and activate i. The active i subunit (i-GPPP) directly inhibits adenylyl cyclase activity. An RGS protein binds to i-GPPP and accelerates the hydrolysis of GTP to GDP resulting in the inactivation of i and the return of the G protein to its heterotrimeric, inactive state (i).

pheromone) that accelerates the hydrolysis of GTP on the Gα subunit and thus desensitizes downstream events that are initiated by activation of the pheromone receptor. Following its discovery in yeast, homologues of Sst2p were found in mammalian cells (De Vries et al., 1995). The mammalian homologues of Sst2p are termed Regulators of G protein Signalling (RGS) proteins. The RGS proteins accelerate the hydrolysis of GTP bound to various G protein α subunits. Today, about 30 RGS proteins have been identified in mammalian tissues (De Vries et al., 2000).

RGS proteins accelerate GTP hydrolysis

The intrinsic GTPase activity of the G protein α subunit is a cellular clock, terminating receptor signalling by returning the G protein to its inactive, GDP-bound state (Huang et al., 1997; Druey et al., 1998). RGS proteins increase the rate of GTPase activity by binding to the α subunit. The interaction of an RGS protein with a G protein greatly decreases the period of time that the G protein is active, thereby attenuating signalling. All RGS proteins share a region of approximately 120 amino acids (the RGS domain) that binds to the GTP-bound Gα subunit and accelerates its rate of GTP hydrolysis by at least 40-fold (De Vries et al., 1995; Druey et al., 1996; Dohlman and Thorner, 1997) (Berman et al., 1996a; Neubig and Siderovski, 2002). X-ray crystallography studies reveal that the RGS domain of RGS4 contacts the three switch regions of Gαi1 and stabilizes the transition state between GTP-bound and GDP-bound forms of the Gαi1 (Tesmer et al., 1997). The majority of RGS proteins interact with Gαi, Gαo, and Gαq, but not with Gαs or Gα12 (Berman et al., 1996a; Hunt et al., 1996).

Only one RGS protein, RGS-PX1, has been found to interact with G□s and to diminish G□s-mediated stimulation of adenylyl cyclase activity (Zheng et al., 2001). Recombinant RGS proteins (such as RGS4) attenuate somatostatin receptor-mediated inhibition of cyclic AMP accumulation (Huang et al., 1997) and increase muscarinic agonist-mediated GTPase activity (Cladman and Chidiac, 2002). Thus, RGS proteins are capable of diminishing the signalling of receptors coupled to Gi-type G proteins in intact cells.

Evidence that endogenous RGS proteins play a physiological role in G protein-coupled receptor (GPCR) signalling is beginning to emerge. In the vertebrate rod phototransduction, light activates rhodopsin (the GPCR), and activated rhodopsin catalyzes the exchange of GTP for GDP on the □ subunit of the G protein, transducin (Arshavsky and Pugh, 1998). The GTP-bound form of transducin stimulates the enzyme cGMP phosphodiesterase (PDE) to hydrolyze cGMP to GMP. The hydrolysis of cGMP to GMP results in closure of Na^+ and Ca^{2+} ion channels and hyperpolarizes the rod cell. Hydrolysis of GTP to GDP by the intrinsic GTPase of transducin inactivates PDE. For years the kinetics of the hydrolysis of GTP by transducin was studied in cell-free systems. In these systems the rate of hydrolysis was much slower than those seen in intact rod cells. He *et al.* (1998) found that endogenous RGS9 was the predominant RGS protein expressed in the retina. When RGS9 was combined with transducin and PDE, the rate of GTP hydrolysis was consistent with kinetics observed in the intact rod. In agreement with these findings, Chen *et al.* (2000) reported that

photoreceptors within the retina of RGS-9 deficient mice showed greatly slowed inactivation of the photon-induced signalling cascade.

Other evidence that endogenous RGS proteins play a role in GPCR signalling has been found using mutant forms of G proteins that are insensitive to RGS proteins. DiBello *et al.* (1998) first demonstrated that a single point mutation (glycine to serine) in the yeast Gα subunit Gpa1 prevents the endogenous RGS protein (Sst2p) from stimulating the GTPase of Gpa1 (DiBello et al., 1998). A similar single point mutation in Gα and Gαi results in mutant G protein α subunits with GDP release and GTP hydrolysis kinetics similar to wild type, but the mutation renders the Gα subunit insensitive to RGS proteins (Lan et al., 1998). Expression of RGS-resistant Gαo markedly increases the potency of norepinephrine in closing Ca^{2+} channels (Jeong and Ikeda, 2000), and increases mu opioid-agonist-mediated inhibition of adenylyl cyclase activity in C6 glioma cells (Clark et al., 2003). These studies suggest that endogenous RGS proteins routinely play a role in turning off GPCR signalling by accelerating the rate of GTP hydrolysis by G proteins.

RGS4 as a potential regulator of the mu opioid receptor

RGS proteins are likely to play a role in normal mu receptor signalling and in the development of tolerance to opiates. A subset of RGS proteins have been shown to increase the GTPase activity of Gi-types of G proteins, the types of G proteins that mediate the downstream effects of the mu opioid receptor (Berman et al., 1996a; Hunt et al., 1996; Hepler et al., 1997; He et al., 1998; Ingi et al.,

1998). Of the RGS proteins that have been shown to interact with Gi/Go type G proteins, six (RGS2, RGS4, RGS5, RGS7, RGS9-2, and RGS10) are expressed in the central nervous system (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002). Of these six, only RGS2 and RGS4 mRNA have been detected in most of the brain regions that express mu opioid receptors (Mansour et al., 1995; Gold et al., 1997). Although the mRNA distributions of RGS2 and RGS4 are similar, RGS2 mRNA is absent in certain regions of the brain that contain high levels of mu receptors and RGS4, notably the amygdala, medial habenula, hypothalamus, and ventral tegmental area. Furthermore, RGS2 preferentially interacts with G□q (Ingi et al., 1998; Neubig and Siderovski, 2002). The distribution and functional specificity of RGS4 make it the most likely of the RGS proteins to be involved in mu opioid receptor functioning and desensitization.

Nakagawa *et al.* (2001) demonstrated that treatment of PC12 cells (stably transfected with mu receptors) with morphine for 0.5 - 24 h increases RGS4 mRNA levels 140-170 % (Nakagawa et al., 2001). Also, *in situ* hybridization studies in the lumbar spinal cord show that, during the development of chronic neuropathic pain and hyperalgesia, RGS4 is the only RGS protein whose mRNA levels increase significantly (230%). In contrast, messenger RNA levels of GAIP, RGS6, RGS7, RGS8, RGS9, RGS11, RGS12, RGS14 and RGS17 are not elevated (Garnier et al., 2003). Overexpression of RGS4 in HEK 293 cells attenuates mu receptor-mediated inhibition of adenylyl cyclase activity (Garnier et al., 2003). These data support the view that morphine insensitivity following

prolonged use may be a result of an increase in the amount or subcellular location of RGS4.

The specific expression and distribution of endogenous RGS proteins in the brain is not yet known due to the lack of antibodies that are capable of detecting these proteins in immunohistochemical studies. RGS4 interacts primarily with Gi and Go types G proteins (Berman et al., 1996b), the same G proteins that associate with mu receptors (Chalecka-Franaszek et al., 2000). Also, RGS4 enhances the GTPase activity of these G proteins (Berman et al., 1996a). Because morphine does not cause sufficient □-arrestin association with the mu receptor to cause internalization of the mu receptor (Zhang et al., 1998), another mechanism is most likely involved with the development of tolerance to morphine.

Significance of proposed studies

The subject of this doctoral dissertation research is to investigate the role of RGS4 on mu opioid receptor signalling. RGS4 may contribute to the development of tolerance following chronic opiate administration by becoming associated with the G proteins that mediate the downstream effects of mu opioid-receptor activation.

The proposed studies were designed to determine whether RGS4 plays a role in the development of tolerance to mu opioid agonists.

Hypothesis: 1. Chronic activation of the mu receptor causes RGS4 to translocate from the nucleus to the cytosol. 2. Chronic mu receptor activation

causes RGS4 to associate with the receptor, bringing RGS4 in close proximity to the receptor-associated Gi-type G proteins, resulting in an increase in the GTPase of these G proteins.

To evaluate the role of RGS4 in mu opioid receptor desensitization, the following specific aims were investigated:

1. To determine the preincubation time necessary for enhancing the ability of RGS4 to blunt mu receptor-mediated inhibition of adenylyl cyclase activity.

2. To determine how chronic DAMGO treatment enhances the efficacy of RGS4 in blunting mu opioid receptor mediated inhibition of adenylyl cyclase activity.

3. To determine if RGS4 and the mu opioid receptor are co-localized in the rat brain and if chronic agonist stimulation causes RGS4 translocation from the nucleus to the cytosol.

An understanding of how chronic mu receptor activation leads to an increased association of RGS proteins with the mu receptor will enable development of pharmacological approaches that will reduce the influence of RGS proteins on mu receptor, allowing for improved analgesics while avoiding the development of tolerance.

This dissertation, "Co-expression of Regulator of G Protein Signalling 4 (RGS4) and the mu opioid receptor in regions of rat brain: Evidence that RGS4 attenuates mu opioid receptor signalling" provides an introduction to the molecular mechanisms of opioid receptor function, the development of tolerance

(associated with prolonged receptor stimulation), and the function of RGS proteins, a family of proteins known to blunt the inhibitory G proteins that mediate G protein-coupled receptor signalling. The introduction is followed by two manuscripts, "Attenuation of mu opioid receptor signal transduction by recombinant RGS4", submitted to *The Journal of Neurochemistry*, and "Co-expression of the mu opioid receptor and RGS4 protein in regions of the rat brain: *Fentanyl treatment affects RGS4 sub-cellular location",* submitted to the *Journal of Biological Chemistry.* The first paper provides pharmacological data that support a role for a particular RGS family member, RGS4, in the development of mu opioid receptor tolerance. The second paper provides immunohistochemical evidence that RGS4 is found in regions of rat brain that also express high levels of the mu opioid receptor. Following the manuscripts is a summary and discussion of the results, as well as future directions. Finally, an addendum contains data from, and discussions of, experiments that yielded negative results.

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Chapter 2

Attenuation of mu opioid receptor signal transduction by recombinant RGS4

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Abbreviations: RGS, regulator of G protein signalling; DAMGO, [D-Ala^2, N-Me-Phe^4, gly-ol] enkephalin; GTPγS, guanosine 5'-O-(3-thiotriphosphate); GRK2, G protein receptor kinase II; GAP, guanosine triphosphatase-activating protein; DMEM, Dulbecco's Modified Eagle Medium; GTPase, guanosine triphosphatase; DTT, dithiothreitol.

SUMMARY

Regulators of G protein signalling (RGS) proteins influence G protein-coupled receptor signal transduction by enhancing the intrinsic guanosine triphosphatase (GTPase) activity of G protein α subunits. The RGS-enhanced GTPase activity of Gα subunits may be responsible for the desensitization of certain G protein-coupled receptors, including the mu opioid receptor. Because RGS4 mRNA occurs in most brain regions that express mu opioid receptors, we evaluated the ability of recombinant RGS4 to affect the mu receptor agonist [D-Ala^2, N-Me-Phe^4, gly-ol] enkephalin (DAMGO)-mediated inhibition of adenylyl cyclase activity in SH-SY5Y cell membranes. Recombinant RGS4 caused a concentration-dependent attenuation of DAMGO-mediated inhibition of adenylyl cyclase activity (EC_{50} 0.19 ± 0.1 μM). RGS4 diminished the efficacy, but not the potency, of DAMGO in inhibiting adenylyl cyclase activity. In contrast, RGS4 did not affect the ability of guanosine 5'-O-(3-thiotriphosphate) (GTPγS) to inhibit adenylyl cyclase activity or the ability of [^{3}H]DAMGO to bind to the mu opioid receptor. We also determined that RGS4 does not affect the ability of DAMGO to stimulate [^{35}S]GTPγS binding to SH-SY5Y membranes. These findings are consistent with the hypothesis that RGS4 can negatively regulate mu opioid receptor signal transduction by increasing the intrinsic GTPase of its associated Gi-type G subunits that mediate agonist action through effector pathways. **Key Words:** mu opioid receptor, signal transduction, RGS4, G protein, adenylyl cyclase.

INTRODUCTION

Opiates are the most effective analgesics for the relief of severe pain. Unfortunately, clinical use is limited by the development of tolerance, a progressive decrease in potency and efficacy that results in diminished analgesia. The ability of a receptor to down-regulate its response to chronic stimulus has been extensively characterized. Despite many recent advances in understanding the cellular adaptations resulting from chronic opiate use, including changes in the number and function of guanine nucleotide binding proteins (G-proteins) (Strassheim and Malbon, 1994), and compensatory upregulation of adenylyl cyclase (Nestler, 1993), the molecular events responsible for the development of tolerance remain unresolved.

Mu opioid receptor desensitization has been widely used as a model for the development of tolerance. In HEK 293 cells, transiently transfected with mu opioid receptors, chronic exposure to an opiate agonist, etorphine, diminished the ability of subsequent opiate exposure to inhibit adenylyl cyclase activity (Zhang et al., 1998). In these studies, agonist occupancy of the mu receptor caused G protein receptor kinase II-mediated (GRK2) receptor phosphorylation. Once the receptor was phosphorylated, □arrestin translocated to the plasma membrane and uncoupled the receptor from its cognate G protein (Krupnick et al., 1997; Carman and Benovic, 1998). □arrestin promotes receptor internalization by acting as an "adapter" linking the receptor to clathrin-coated pits. This process is followed by subsequent dynamin-mediated receptor internalization (Lohse et al., 1990; Zhang et al., 1998; Goodman et al., 1996; Nakamura et al., 1998). After internalization,

the receptor may either be dephosphorylated and returned to the cell surface or degraded. Together, the results of these studies suggest that desensitization occurs in two general phases. Initially (within minutes), there is a diminished ability of the receptor to activate its cognate G protein as a result of the binding of arrestin to the receptor, and later (over a number of hours), there is a reduction in the number of receptors resulting from degradation during the receptor internalization event. However, opiate tolerance can develop in the absence of the aforementioned processes. For example, even though morphine increases mu opioid receptor phosphorylation, it does not activate GRK2, and causes neither an increase in receptor internalization nor a decrease in receptor number, despite the fact that chronic administration of morphine results in the development of tolerance (Deng et al., 2001; Patel et al., 2002). Thus, chronic morphine treatment brings about changes in neurons expressing mu receptors that impact mu receptor signal transduction without affecting mu receptor number or agonist binding.

The standard model of G-protein coupled receptor signal transduction proposes that agonist-induced conformational changes in the receptor result in an enhanced release of GDP, followed by the subsequent binding of GTP, by the -subunit of the G-protein heterotrimer (Gilman, 1987). In this model, the duration of signalling is dictated by the lifetime of the subunit in the GTP-bound form. Once GTP is hydrolyzed via an intrinsic GTPase activity, signalling is terminated. The rate of GTP hydrolysis by the G-protein alone is too slow to account for the rapid termination of signalling seen in physiological systems (Berman and Gilman,

1998). Therefore, the discovery of **G**uanosine triphosphatase-**A**ctivating **P**roteins (GAPs), intracellular proteins which serve to accelerate GTP-hydrolysis, was an important development in the study of G-protein coupled receptor function (Scheffzek et al., 1998; Donovan et al., 2002).

Of particular interest was the discovery of GAPs for G subunits termed RGS proteins (for Regulators of G protein Signalling). The first of these proteins, Sst2p, discovered in *Saccharomyces cerevisiae*, was found to desensitize pheromone receptor signalling (Dohlman et al., 1995). Following its discovery, approximately 30 mammalian homologues of Sst2p have been identified (Hepler, 1999; De Vries et al., 2000; Neubig and Siderovski, 2002). Many of these RGS proteins have been proposed to negatively regulate receptors that activate inhibitory G proteins by acting as GAPs toward G subunits, increasing the rate of GTP hydrolysis by at least 40-fold (Berman et al., 1996b). Though RGS proteins are a highly diverse, multifunctional family of proteins, differing in size, composition, and distribution, they all share a highly conserved 120 amino acid region, termed the "RGS box" or "RGS homology domain" (Gold et al., 1997; Grafstein-Dunn et al., 2001) (Popov et al., 1997). Truncation studies indicate that the RGS box contains all the elements necessary for the GTPase-activating function, and the N-terminal and C-terminal regions contain additional domains that link RGS proteins with specific signalling pathways (De Vries et al., 1995; De Vries et al., 2000).

The goal of the current study was to determine if an RGS protein could contribute to the development of tolerance of the mu opioid receptor. Of the

nearly 30 mammalian RGS proteins, RGS4 appeared to be a good candidate to play a role in the development of mu receptor tolerance. *In situ* hybridization studies demonstrate that RGS4 mRNA is present in high levels in many of the same areas of the brain that express mu opioid receptors (Mansour et al., 1995a; Gold et al., 1997; Nomoto et al., 1997; Ingi and Aoki, 2002). Additionally, RGS4 protein interacts with Gαi1, Gαi3, and Gαo protein subunits, the same subunits we found to co-immunoprecipitate with the activated mu opioid receptor (Chalecka-Franaszek et al., 2000). Further, morphine increased RGS4 mRNA in PC12 cells that were transfected with mu receptors (Nakagawa et al., 2001). Together, this accumulated evidence suggests that RGS4 may play a role in the development of agonist-induced desensitization of the mu opioid receptor.

In the current study, recombinant RGS4 was tested for its ability to affect mu opioid receptor-mediated inhibition of adenylyl cyclase activity. It was discovered that the addition of RGS4 to broken cell preparations diminished the efficacy, but not the potency of DAMGO in inhibiting adenylyl cyclase activity. In contrast, RGS4 did not affect GTPγS-mediated inhibition of adenylyl cyclase activity, [^{3}H]DAMGO binding to the mu receptor, or the ability of DAMGO to stimulate GTPγS binding to Gi-type G proteins. These findings are consistent with the proposal that RGS4 may attenuate mu opioid receptor-mediated inhibition of adenylyl cyclase activity by enhancing the GTPase activity of the Gi-type G proteins associated with the mu opioid receptor.

MATERIALS AND METHODS

Drugs and Cell Culture. [D-Ala^2, N-Me-Phe^4, gly-ol] enkephalin (DAMGO) was purchased from Sigma, penicillin/streptomycin, G418, and Glutamax 1 from Gibco Life Technologies BRL (Rockville, MD), [^{3}H]cAMP from NEN Life Sciences, and [^{3}H]DAMGO from Amersham Pharmacia (Piscataway, NJ). [^{35}S]GTPγS was obtained from Perkin Elmer Life Sciences (Boston, MA). Undifferentiated SH-SY5Y neuroblastoma cells were purchased from American Type Cell Culture (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (CellGro), supplemented with 15% fetal calf serum, 1 mM Glutamax, 100 units/ml penicillin, 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 C.

Preparation of recombinant RGS4. Full length GST-RGS4 was prepared by subcloning RGS4 from pQE60 into pGEX2T as previously described (Chalecka-Franaszek et al., 2000). The following primers were used to amplify full-length rat RGS4 from a template of RGS4 pQE60 cDNA, a gift from Susan Mumby.

5’-CGCG **GGA TCC** ATG TGC AAA GGA CTC GCT GGT CTG-3' (sense)

5’-GCGC <u>GAA TTC</u> TTA GGC ACA CTG AGG GAC TAG GGA-3' (antisense)

Our sense primer and antisense primer have BamH1 and EcoR1 restriction sites at their 5’ ends, indicated by bold print and underline, respectively. A stop codon was placed in the antisense primer to ensure that the C-terminal of the fusion

protein would be identical to that of the rat RGS4 protein. PCR primer extension, using these primers, resulted in the generation of a 618 base pair product.

The purified PCR products were digested with *Bam*H1 and *Eco*R1 endonucleases and ligated into a linearized fusion protein expression vector (pGEX-2T). Competent JM109 *E. coli* bacteria (Promega; Madison, WI) were transformed with the pGEX-2T plasmids containing the PCR-generated DNA. Plasmids were isolated from transformants using the Quantum Prep Plasmid Midiprep Kit (Bio-Rad, Hercules, CA). Each isolated plasmid was then used as the template for automated fluorescent DNA sequencing in both the 5' and the 3' directions to verify proper in-frame ligation of the RGS4 product and to verify that no mutations had been introduced. The construct, encoding all 205 amino acids of native RGS4 fused to GST was transformed into *E. coli* strain JM109.

An overnight culture of bacteria (5 ml) containing the GST-RGS4 plasmid was diluted into 500 ml Luria broth containing ampicillin (100µg/ml) and incubation was continued for approximately 1.5 h. When the OD_{600} of the culture reached 0.60 - 0.80, fusion protein expression was induced by the addition of 1.0 mM isopropanol □-D-thiogalactosidase, and the incubation was continued for 3 h at 37 C. The GST-RGS4 fusion protein was extracted as described previously (Chalecka-Franaszek et al., 2000) with the following modification. Bacterial supernate (25 ml) containing the GST-RGS4 fusion protein product was incubated on a Lab Quake with 2 ml of a 1:1 slurry of Glutathione Sepharose 4B beads presorbed in 20 mM Hepes (pH 7.6), 100 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT) (Buffer C) for 30 min at room temperature. Beads were

centrifuged at 500 x g for 5 min, the pellets were washed five times with excess Buffer C. The washed beads were resuspended in 5 ml Buffer C and 10 units of thrombin protease/mg protein (Amersham Biosciences; equivalent to 1 NIH unit/mg protein) and incubated in a 30 C water bath with gentle agitation for 4 h. GST remained bound to the glutathione agarose beads while RGS4 entered the supernatant. The sample was centrifuged at 500 x g for 5 min and the supernatant, containing the RGS4 protein, was concentrated to 0.5 mg protein/ml with a Centriprep 30 Centrifugal Filter (Amicon; Bedford, MA).

Preparation of SH-SY5Y cells. SH-SY5Y cells were grown in 75 mm flasks to 80% confluency in Dulbecco's Modification of Eagle's Medium containing 4.5 g/l glucose, 1% Glutamax, penicillin (100 units/ml), streptomycin (100 µg/ml), and 15% fetal calf serum (DMEM). Routinely, cells were pretreated for 2 h with 1 µM DAMGO prior to their use in all assays because, in initial experiments, pretreatment with DAMGO modestly enhanced the ability of recombinant RGS4 to attenuate mu opioid receptor signalling by about 10-15% although this enhancing effect was not always significant. Furthermore, it was reasoned that exposure of cells to a mu agonist might modify the receptor in such a way that the receptor would more effectively interact with the RGS protein. After the 2 h exposure to DAMGO, the cells were rinsed and incubated an additional 30 min in DMEM without DAMGO prior to their use in various assays. The 2 h DAMGO pretreatment alone had no effect on subsequent DAMGO-mediated inhibition of adenylyl cyclase activity, [^{3}H]DAMGO binding, or mu receptor-stimulated

[^{35}S]GTPγS binding to membranes. Thus, in all the experiments performed in this study, SH-SY5Y cells were pretreated for 2 h with 1 μM DAMGO prior to their use in the adenylyl cyclase assay, the [^{3}H]DAMGO binding assay, or the mu receptor-stimulated [^{35}S]GTPγS binding assay.

Adenylyl cyclase assay. SH-SY5Y cells were washed three times in PBS, and scraped from the plate. The cell suspension was centrifuged at 400 x g for 2 min, and the resulting cell pellets were resuspended in homogenizing buffer (20 mM Tris·HCl (pH 7.4), 2 mM EGTA, 1 mM $MgCl_2$, 250 mM sucrose) to attain 0.2 g wet weight/ml and homogenized with 6 strokes of a glass-Teflon Dounce homogenizer (Wheaton; Philadelphia, PA). Adenylyl cyclase activity was measured as described previously (Cote et al., 1981; Frey and Kebabian, 1984; Aub et al., 1986; Puttfarcken et al., 1986; Gosse et al., 1989). Cells were pretreated with 1 μM DAMGO as described above prior to their use in the adenylyl cyclase assay. Briefly, the assay was performed in a final volume of 60 μl containing 80 mM Tris·HCl (pH 7.4), 10 mM theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 10 μM forskolin, 10 μM GTP (GTP was omitted when GTPγS was tested), and approximately 15-25 μg of protein from SH-SY5Y cell homogenates. Cell homogenates and the components of the assay (except for ATP, guanine nucleotides and DAMGO) were incubated, with or without RGS4, on ice for 10 min prior to the assay. The ATP, guanine nucleotides, and agonist were added to the components on ice, and the adenylyl cyclase reaction was initiated by placing the tubes in a water bath at 30 C. The activity was terminated

after 10 min by placing the tubes in boiling water for 2 min. The amount of cAMP formed was determined by the protein binding assay of Brown *et al.* (Brown et al., 1971).

DAMGO-stimulated [^{35}S]GTPγS binding to SH-SY5Y cell membranes. DAMGO-stimulated [^{35}S]GTPγS binding to SH-SY5Y cell membranes was performed as previously described (Childers, 1991; Traynor and Nahorski, 1995). Prior to the binding assay, the cells were pretreated for 2 h with 1 μM DAMGO as described above. SH-SY5Y cells were then washed three times in phosphate buffered saline, lifted off the flask surface by scraping, and centrifuged for 5 min at 200 x g. The cell pellet was resuspended in 10 mM Tris·HCl (pH 7.4), homogenized in glass -Teflon Dounce homogenizer and centrifuged at 20,000 x g for 20 min. The pellet was resuspended in 10 mM Tris·HCl (pH 7.4) at 0.15 mg wet weight/ml. Aliquots of crude membrane were added to assay buffer containing 50 mM Tris·HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and preincubated with or without 1 μM RGS4 on ice for 10 min. DAMGO-stimulated [^{35}S]GTPγS binding was performed in a final volume of 100 μl containing 200-300 μg protein/ml of SH-SY5Y membranes, various concentrations of DAMGO (indicated in figure), 10 μM GDP, and 80 pM [^{35}S]GTPγS (specific activity 1250 Ci/mmol). Non-specific binding was defined as binding that occurred in the presence of 10 μM GTPγS. Reaction tubes were incubated at 25 C for 60 min. The assay was terminated by rapid filtration on GF/B filters under vacuum. The filters were washed 3 times with ice-cold assay

buffer. Bound radioactivity was determined after the addition of 3.5 ml of scintillation fluid by counting on a Beckman liquid scintillation counter (Fullerton, CA).

Binding of [^{3}H]DAMGO to mu receptors on SH-SY5Y cell membranes.

[^{3}H]DAMGO binding to mu opioid receptors on SH-SY5Y cell membranes was performed as previously described (Kazmi and Mishra, 1987). Cells were pretreated with 1 µM DAMGO for 2 h prior to the [^{3}H]DAMGO binding assay as described above. SH-SY5Y cells were centrifuged at 500 x g for 5 min, and pellets were resuspended and homogenized in buffer containing 2 mM Tris·HCl (pH 7.4) and 2 mM EGTA. Homogenates were centrifuged for 30 min at 37,000 x g, and the resulting pellet was resuspended in buffer containing 50 mM Tris HCl (pH 7.4), 2 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$ (TEM buffer). The crude membranes were incubated either in the absence or the presence of 1 µM RGS4 on ice for 10 min. Aliquots (100 µl) containing 100-150 µg protein were incubated in the presence of various concentrations of [^{3}H]DAMGO, for 1 h at 30 C. Ten µM morphine was included to determine nonspecific binding for each concentration of [^{3}H]DAMGO. The binding assay was terminated by collecting the samples on GF/B filters under vacuum. Filters were subsequently washed three times with ice-cold TEM buffer, and bound radioactivity determined 16 h after the addition of 3.5 ml of scintillation fluid by counting on a Beckman liquid scintillation counter (Fullerton, CA).

Analysis of Data. All statistical and curve-fitting analyses were performed using PRISM v2.0b for Macintosh (GraphPad Software, San Diego, CA). Non-linear regression analysis was used to determine the best fit of full concentration effect curves for adenylyl cyclase, [^{3}H]DAMGO receptor binding and [^{35}S]GTPγS binding assays. The EC_{50} and B_{MAX} were determined from best fit analysis. The unpaired Student's t test was used to determine statistical significance between means in the adenylyl cyclase assay. Data are expressed as mean ± standard error of the mean and are represented by a minimum of three separate experiments, performed in triplicate or quadruplicate.

RESULTS

Recombinant RGS4 blunts DAMGO-mediated inhibition of adenylyl cyclase activity.

We determined whether recombinant RGS4 affected mu opioid receptor signalling in SH-SY5Y cells, a human neuronal cell line expressing endogenous mu receptors. Recombinant RGS4 was produced in and purified from *E. coli* as described in methods. The predominant product ran as a single band of ~25 kDa on a 12% SDS-PAGE. Routinely, the 25 kDa band made up 60-80% of all protein stained with Commassie blue (Fig. 1). The 25 kDa band was specifically identified in a Western blot with our antibodies raised against the C-terminal 36 amino acids of RGS4 (GST-RGS4$_{170\text{-}205}$) (not shown).

Isolated, recombinant RGS4 protein was tested for its ability to diminish mu receptor-mediated inhibition of adenylyl cyclase activity in freshly prepared homogenates of SH-SY5Y cells that had been treated with 1 μM DAMGO for 2 h prior to harvest. Preliminary experiments indicated that a 2 h pretreatment with DAMGO modestly enhanced the RGS4 effect, although this effect was not consistently significant. Nevertheless, we routinely pretreated cells with DAMGO for 2 h prior to experimental procedures to mimic potential changes that may occur to the mu receptor during chronic exposure to opiates. Importantly, the 2 h pretreatment did not affect subsequent DAMGO inhibition of adenylyl cyclase activity in the absence of RGS4. The addition of RGS4 caused a concentration dependent attenuation of DAMGO inhibition of adenylyl cyclase activity (Fig. 2). In three experiments, maximal inhibition of adenylyl cyclase by 1 μM DAMGO was

58 ± 3 % and 21 ± 1 %, in the absence and presence of 1 µM RGS4, respectively. The half-maximal inhibitory effect of RGS4 occurred at a concentration of 0.19 ± 0.09 µM.

RGS4 decreases the efficacy, but not the potency of DAMGO

To further characterize the effect of RGS4 on mu opioid receptor signalling, RGS4 was tested for its ability to diminish the potency or the efficacy of DAMGO in inhibiting adenylyl cyclase activity. DAMGO concentration response curves were generated in homogenates of SH-SY5Y cells in the absence and presence of 1 µM RGS4 (Fig. 3). DAMGO maximally inhibited adenylyl cyclase activity 52 ± 1% and 28 ± 2 %, respectively ($t = 9.55$, $p < 0.01$). The EC50 of DAMGO in inhibiting adenylyl cyclase activity in the presence and absence of 1 µM RGS4 was 20 ± 7 nM and 36 ± 11 nM, respectively ($t = 1.22$, $p = 0.23$, not significant).

To further examine the mechanism of action of RGS4 in diminishing mu receptor-mediated signalling, the effect of RGS4 on inhibition of adenylyl cyclase by a nonhydrolyzable guanine nucleotide, guanosine 5'–*O*-3-thiotriphosphate (GTPγS) was also studied. Various concentrations of GTPγS were tested in the absence or presence of 1 µM RGS4. RGS4 failed to affect GTPγS-mediated inhibition of adenylyl cyclase activity (Fig. 4). In four separate experiments, the EC50 of GTPγS was 41.7 nM ± 13 and 34.0 ± 12 nM in the absence and presence of RGS4, respectively. Additionally, in the absence and presence of RGS4, 1 µM GTPγS caused a 57 ± 6.3 % and 62 ± 8.1 % inhibition of adenylyl cyclase, respectively. It is widely appreciated that RGS proteins act to increase the rate of

GTP hydrolysis by stabilizing the transition state of the Gα subunit (Tesmer et al., 1997). This view is further supported by the present data showing that RGS4 does not affect the potency or the efficacy of GTPγS inhibition of adenylyl cyclase.

The ability of RGS4 to affect mu receptor-mediated stimulation of GTPγS binding to Gi also was investigated. Increasing concentrations of DAMGO were used to stimulate [^{35}S]GTPγS binding to SH-SY5Y membranes in the absence and presence of 1 µM RGS4, as shown in Figure 5. RGS4 failed to affect DAMGO-stimulated [^{35}S]GTPγS binding to the membranes. DAMGO maximally stimulated [^{35}S]GTPγS binding 130 ± 7.7 % and 100 ± 6.9 % above basal binding, in the absence and presence of 1 µM RGS4, respectively. The EC50 of DAMGO was 86 ± 47 nM in the absence of RGS4 and 33 ± 15 nM in the presence of 1 µM RGS4. These data indicate that the addition of RGS4 does not affect the ability of DAMGO to stimulate GTP binding to Gi-type G proteins, further supporting the notion that the effect of RGS4 is not to prevent GTP binding, or prevent GDP release from Gi, but rather to enhance GTPase activity associated with Gi-type proteins.

RGS4 does not affect receptor-ligand interactions

To determine whether the observed blunting of agonist-mediated inhibition of adenylyl cyclase activity was a result of a change in ligand/receptor interactions, RGS4 was tested for its ability to affect [^{3}H]DAMGO binding. RGS4 failed to affect either the K_D or the B_{max} of [^{3}H]DAMGO in saturation binding experiments (Fig. 6). Maximal [^{3}H]DAMGO binding in the absence of RGS4 was 58.8 fmol/mg

protein and 58.2 fmol/mg protein in the presence of 1 μM RGS4. These data indicate that the ability of RGS4 to blunt mu receptor mediated inhibition of adenylyl cyclase activity is not a result of changes in the number of receptors or changes in the affinity of the receptor for its ligand.

RGS4 does not associate directly with the mu opioid receptor

Since RGS4 attenuated mu receptor-mediated inhibition of adenylyl cyclase activity, an attempt was made to determine if RGS4 could physically associate with the mu opioid receptor. Recombinant RGS4 (0.3 μM) was mixed with rat brain membranes (from both control and fentanyl-treated rats). The membranes were then solubilized in CHAPS detergent, and mu receptors were immunoprecipitated from the solubilized membrane preparation using antibodies against the mu opioid receptor as previously described (Chalecka-Franaszek et al., 2000). The immunoprecipitated material was subjected to Western blot analysis using antibodies against the C-terminal 50 amino acids of the mu receptor and with antibodies directed towards the C-terminal 36 amino acids of RGS4. Although mu opioid receptors were identified in the Western blots, no recombinant RGS4 was detected in the pellet even though the RGS4 antibodies were capable of detecting as little as 1 ng of recombinant RGS4 (data not shown). Therefore, it appears that RGS4 does not form a strong physical association with the mu opioid receptor.

DISCUSSION

RGS proteins are likely to play a role in the development of tolerance to opiates. A subset of RGS proteins have been shown to increase the GTPase activity of Gi-type G subunits, the type of G proteins that mediate the downstream effects of the mu opioid receptor (Berman et al., 1996b; Hunt et al., 1996; Hepler et al., 1997; He et al., 1998). Of the RGS proteins that interact with Gi/Go, six (RGS2, RGS4, RGS5, RGS7, RGS9-2, and RGS10) are expressed in the central nervous system (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002). Of these six, only RGS2 and RGS4 mRNA have been detected in most of the brain regions that express mu opioid receptors (Mansour et al., 1995b; Gold et al., 1997; Taymans JM, 2002). Though the mRNA distribution of RGS2 and RGS4 are similar, RGS2 mRNA and/or immunoreactivity is absent in certain regions of the brain that contain high levels of mu receptors and RGS4, notably the amygdala, medial habenula, hypothalamus, and ventral tegmental area (Taymans JM, 2002). Furthermore, RGS proteins demonstrate clear specificity for G subfamilies and unlike RGS4, RGS2 preferentially interacts with Gq (Ingi et al., 1998; Neubig and Siderovski, 2002). The distribution and functional specificity of RGS4 make it the most likely of the RGS proteins to be involved in mu opioid receptor desensitization.

The current study was conducted to determine if RGS4 could play a role in modulating mu opioid receptor signalling. Previous studies have characterized many of the molecular functions of RGS4. Co-immunoprecipitation of the G protein subunits and RGS proteins revealed substantial binding of RGS4 to the

G□i subfamily, and *in vitro* experiments demonstrated that purified, recombinant RGS4 accelerates the GTPase activity of the G□i subfamily, in particular G□i1, G□i3, and G□o, the same subunits that were shown to functionally associate with the mu opioid receptor (Berman et al., 1996a; Dohlman and Thorner, 1997b; Lan et al., 2000); (Chalecka-Franaszek et al., 2000). Additionally, when RGS4 protein was stably expressed in a mammalian cell line, somatostatin-mediated inhibition of adenylyl cyclase activity was attenuated while, in contrast, the isoproterenol-mediated accumulation of adenylyl cyclase was unaffected. These results strongly support the capacity of RGS4 to stimulate the GTPase activity of the Gi, but not the Gs subfamilies of G protein □ subunits (Huang et al., 1997). Finally, RGS4 mRNA was increased following stimulation of the Gi cascade by morphine and DAMGO in PC12 cells that were transfected with mu opioid receptors (Nakagawa et al., 2001). Together, these data support RGS4 as a GAP for Gi-type G-proteins and, in concert with the detection of RGS4 mRNA in regions of the brain that also express mu opioid receptors, support the notion that RGS4 may play a role in the desensitization of the mu opioid receptor.

In this study, we examined the influence of recombinant RGS4 on DAMGO-mediated inhibition of adenylyl cyclase activity. Adenylyl cyclase is an important regulator of neural function whose activity is inhibited by the activation of the mu opioid receptor. The inhibition of adenylyl cyclase activity and the resultant reduction of cAMP due to opioid administration have been extensively studied. RGS4 attenuated mu agonist-mediated inhibition of adenylyl cyclase activity, but did not affect GTP□S mediated inhibition of adenylyl cyclase activity. Both of

these results are consistent with the findings of others demonstrating a primary mechanism of action of RGS4 as a GAP.

Concentration response curves to DAMGO indicated that RGS4 diminished the efficacy, but not the potency of DAMGO. This demonstrated that RGS4 does not act directly at the mu receptor, but rather acts at a downstream site to attenuate mu receptor signalling. Additionally, RGS4 failed to influence high affinity binding of [^{3}H]DAMGO to mu opioid receptors. Because high affinity agonist binding ([^{3}H]DAMGO binding) requires association of the receptor with its interactive G-protein, these results suggested that RGS4 does not interfere with the coupling of the mu receptor with Gi-type G-proteins. Together, these observations directly demonstrate that the ability of RGS4 to attenuate mu receptor-mediated inhibition of adenylyl cyclase activity was not a result of either a direct effect on the mu opioid receptor, or an effect on the coupling between the mu receptor and its associated G-protein.

Because RGS4 could be mediating the desensitizing effect by altering the ability of the mu receptor to enhance GTP binding to its G-proteins, we examined whether RGS4 could diminish mu receptor stimulated [^{35}S]GTPγS binding to SH-SY5Y membranes. It was found that 1 µM RGS4 failed to affect either the potency or the efficacy of DAMGO in stimulating [^{35}S]GTPγS binding to Gα subunits. The rate-limiting step in G-protein activation and in GTPγS binding is the agonist-stimulated release of GDP from Gα subunits, it can therefore be inferred that RGS4 does not affect mu receptor-stimulated release of GDP from Gi or Go-type G-proteins.

The cellular mechanisms involved in the development of mu opioid receptor desensitization following chronic agonist stimulation are complex. Phosphorylation of the mu receptor, as well as several effects of chronic agonist treatment, including receptor adaptations such as internalization, have been proposed to play a role in the development of opiate tolerance (Taylor and Fleming, 2001). However mu agonists differ greatly in their abilities to phosphorylate, activate, and induce endocytosis of the receptor both in transfected cell lines and in brain tissues (Whistler et al., 1999). Morphine, in particular, induces only a modest increase in the phosphorylation state of mu receptors, relative to other agonists, and interestingly, causes neither an increase in receptor internalization nor a decrease in receptor number, despite the fact that chronic administration results in the development of tolerance (Patel et al., 2002). Therefore, it appears that chronic morphine treatment causes desensitization as a result of changes that do not involve a decrease in mu receptor numbers or a decrease in agonist binding, but that do affect mu receptor signal transduction. We demonstrate that RGS4 does not affect mu receptor binding characteristics, but does negatively modulate mu receptor functioning, a characteristic that lends support to its role in the development of tolerance.

Furthermore, we have demonstrated an interaction between mu opioid receptor function and RGS4. Recombinant RGS4 was found to diminish the efficacy, but not the potency of DAMGO in inhibiting adenylyl cyclase activity in SH-SY5Y cell homogenates. The potency of RGS4 in diminishing mu receptor signalling was similar to the reported potency of RGS4 in increasing the GTPase activity of Gi-

type G proteins (Berman et al., 1996a; Watson et al., 1996). RGS4 had no effect on the ability of GTPγS to inhibit adenylyl cyclase activity or on the ability of a mu agonist to enhance [^{35}S]GTPγS binding to membrane-associated G proteins. These findings are consistent with the idea that RGS4 can diminish mu receptor signalling by inactivating the G-proteins associated with the mu opioid receptor.

The development of opiate tolerance may be the result of the diminished capacity of the Gi-type G proteins to mediate downstream effects of the mu receptor. There is no 'unifying hypothesis' to define the development of tolerance (Taylor and Fleming, 2001), probably because tolerance involves many cellular processes, different signalling pathways, as well as cell specific and regional differences in response to chronic agonist stimulation (Nestler et al., 1994; Law and Loh, 1999). RGS proteins may be a unifying element in the development of tolerance. In particular RGS4 may be an important component in the development of mu opioid receptor desensitization, at least in some regions of the brain. It is has been well established that RGS4 interacts with the Gαi family of G proteins (Dohlman and Thorner, 1997a). Further, we have found that RGS4 is found in the same regions of the brain that express high levels of mu opioid receptor, and that treatment with mu agonist results in a change in the subcellular localization of RGS4 (unpublished observations). We envision that chronic agonist stimulation results in an tightly regulated association of RGS4 with the G proteins associated with the mu receptor, bringing about an enhanced hydrolysis of GTP.

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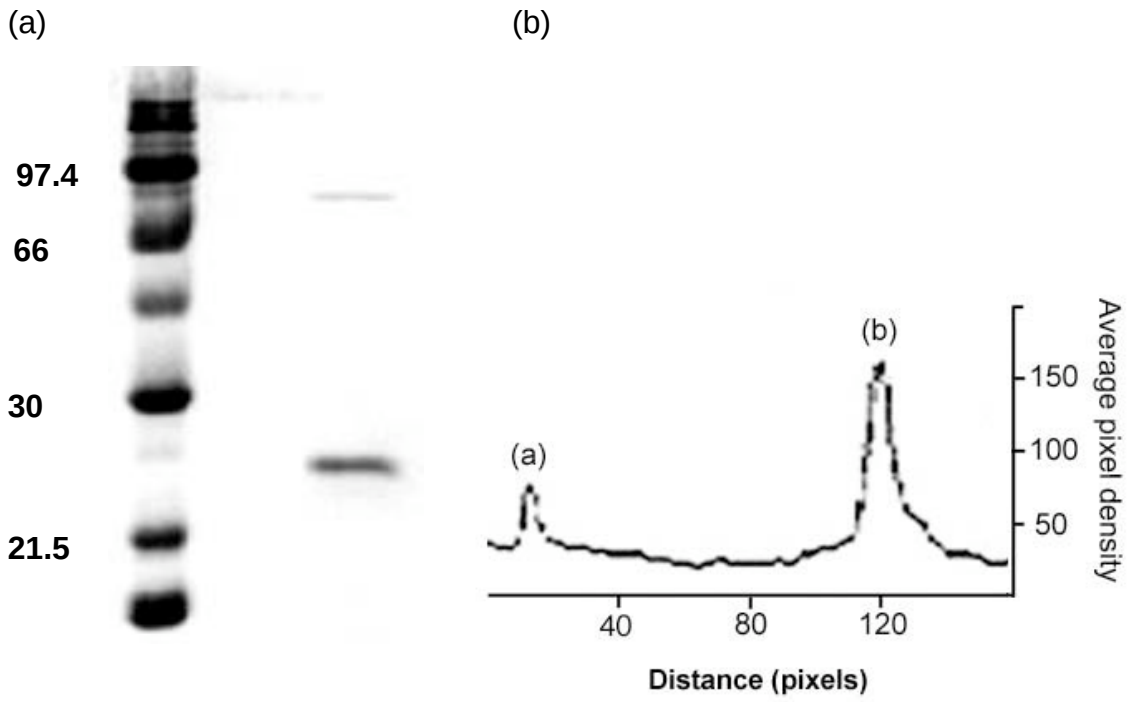

Figure 1. Recombinant RGS4 produced in and purified from *E. coli* runs as an ~ 25 kDa protein on a 12% sodium dodecyl sulfate polyacrylamide gel. Recombinant RGS4 was produced in *E coli*. Bacteria were transformed with the pGEX-2T expression vector containing full length RGS4 cDNA. Recombinant GST-RGS4 fusion protein was purified on glutathione-Sephadex resin and treated with thrombin to cleave GST from RGS4. RGS4 was dialyzed into phosphate buffer saline and subjected to electrophoresis on a 12% SDS-PAGE gel. The gel was stained with Coomassie blue. Left lane shows molecular weight standards as indicated on left (kDa). Right lane shows purified protein. A scan of the right lane indicated that the ~25 kDa band (b) made up 79% of the total Coomassie blue stained protein (shown as (b) in the right lane of the gel.

Figure 2. Concentration-dependent attenuation of DAMGO-mediated inhibition of adenylyl cyclase activity by recombinant RGS4. Forskolin-stimulated adenylyl cyclase activity was measured in SH-SY5Y cell homogenates in the presence of the indicated concentrations of RGS4 alone (open circles) or in combination with 1μM DAMGO (filled circles), 1 μM GTPγS (open diamonds), or 1 μM DAMGO plus 1 μM GTPγS (filled diamonds). Values are expressed as pmoles cAMP/mg protein·min. Membranes were prepared and assessed for adenylyl cyclase activity as described in *Experimental Procedures*. Values in this figure represent the mean ± SEM (n=4) of a single experiment. In three independent experiments the maximal inhibition of adenylyl cyclase activities was 58 ± 3 % and 21 ± 1 % in the absence and presence of RGS4, respectively. In the three experiments the EC_{50} of RGS4 was 0.19 ± 0.09 μM.

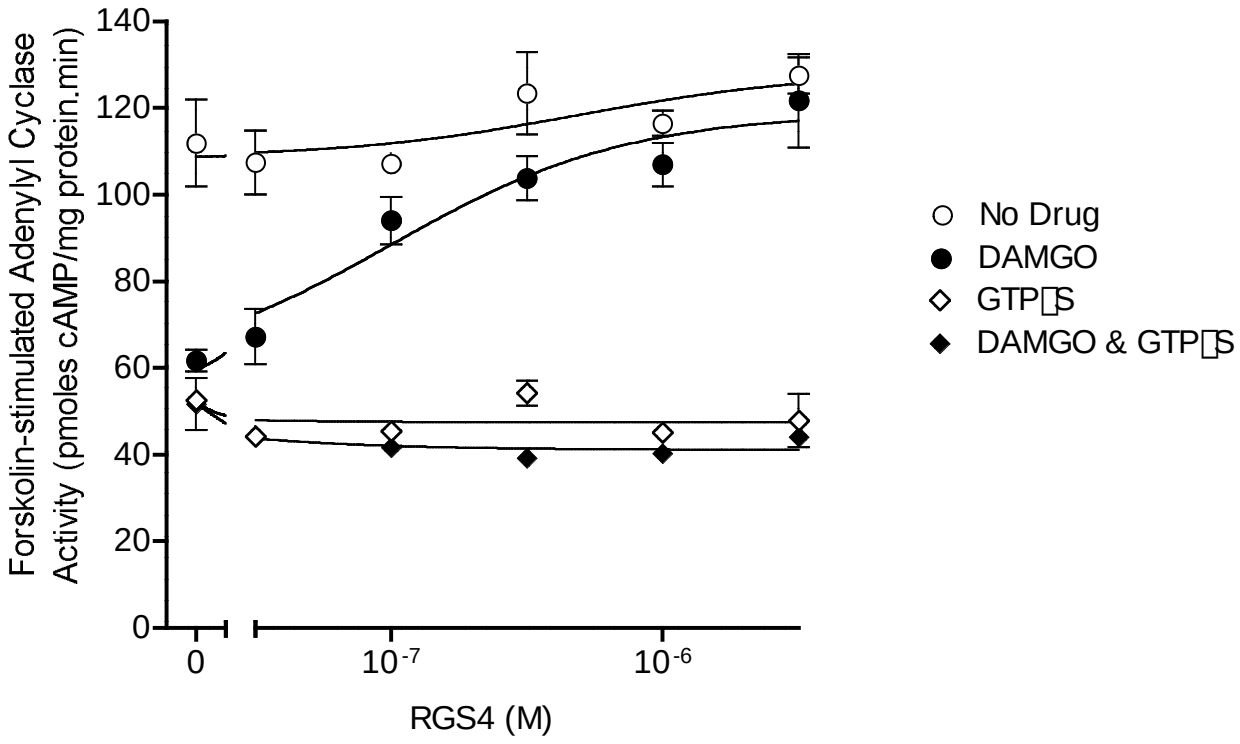
Forskolin-stimulated Adenylyl Cyclase Activity (pmoles cAMP/mg protein.min)
140
120
100
80
60
40
20
0
0
10-7
10-6
RGS4 (M)
No Drug
DAMGO
GTPγS
DAMGO & GTPγS

Figure 2.

Figure 3. RGS4 diminishes the efficacy but not the potency of DAMGO. Forskolin-stimulated adenylyl cyclase activity was measured in SH-SY5Y cell homogenates in the presence of the indicated concentrations of DAMGO alone (open squares) or in combination with 1 µM RGS4 (filled squares). Values are expressed as pmoles cAMP/mg protein·min. In this experiment, DAMGO maximally inhibited adenylyl cyclase activity by 52 ± 1% and 28 ± 2% in the absence and presence of 1 µM RGS4, respectively ($t = 9.55$, $p < 0.00001$). Values represent the mean ± SEM (n=4) of three experiments. In three independent experiments, DAMGO maximally inhibited adenylyl cyclase activity by 57 ± 4% and 21 ± 2% in the absence and presence of 1 µM RGS4, respectively ($t = 7.11$, $p = 0.002$). The potency of DAMGO was unaffected by the addition of RGS4. The EC_{50} values of DAMGO were 20 ± 7 nM and 36 ± 11 nM in the absence and presence of RGS4, respectively ($t = 1.22$, $p = 0.2327$, not significant).

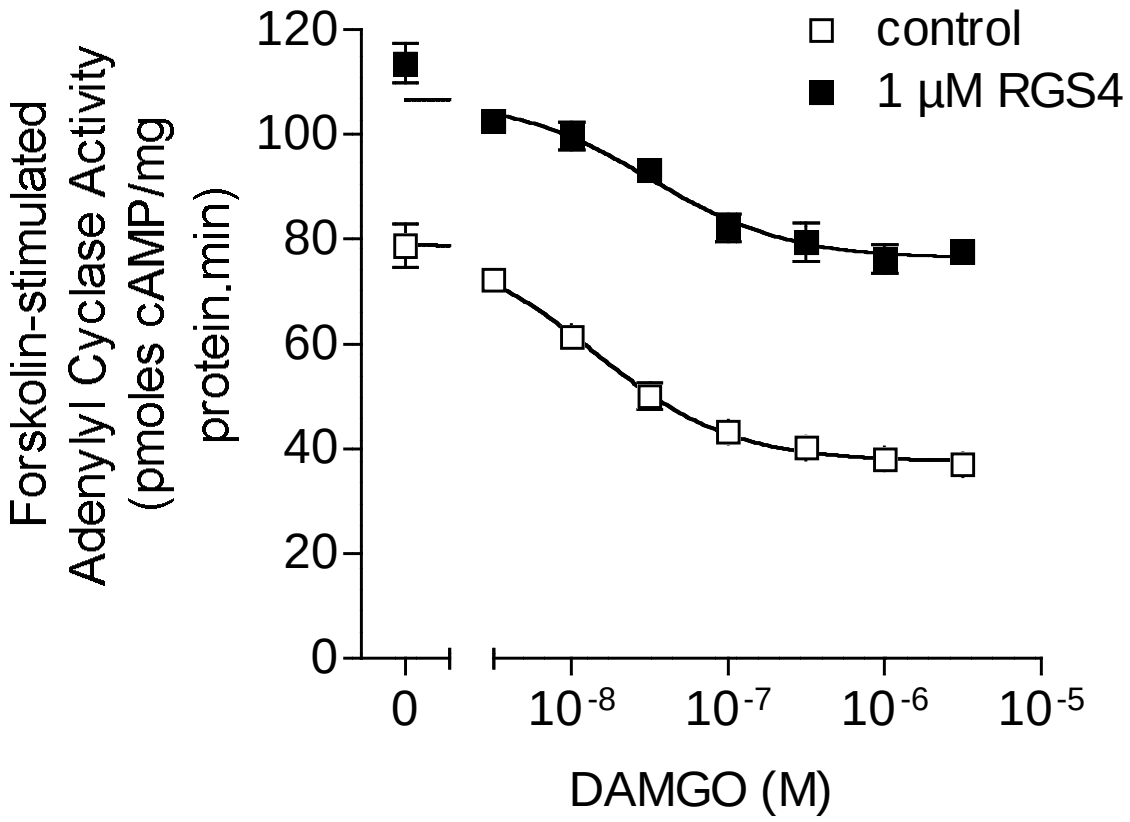
Forskolin-stimulated
Adenylyl Cyclase Activity
(pmoles cAMP/mg
protein.min)
120
100
80
60
40
20
0
control
1 µM RGS4
0
10-8
10-7
10-6
10-5
DAMGO (M)

Figure 3.

Figure 4. RGS4 fails to affect GTPγS-mediated inhibition of adenylyl cyclase. Forskolin-stimulated adenylyl cyclase activity was measured in SH-SY5Y cell homogenates in the presence of the indicated concentrations of GTPγS in the absence (open squares) or presence (filled squares) of 1 μM RGS4. Values are expressed as pmoles cAMP/mg protein·min. In the single experiment depicted in this figure, the means ± SEM of maximal inhibition of adenylyl cyclase activity in the absence and presence of RGS4 were 57 ± 6.3 % and 62 ± 8.1 %, respectively (no significant difference). In four independent experiments the means of the maximal inhibition of adenylyl cyclase activity by GTPγS were 59.1 ± 1.5 and 62.4 ± 5 % in the absence and presence of 1 μM RGS4, respectively ($t = 0.64$, $p = 0.54$, not significant). The EC_{50} values of GTPγS were 41.7 ± 13 nM and 34.0 ± 12 nM in the absence and presence of RGS4, respectively ($t = 0.43$, $p = 0.68$, not significant).

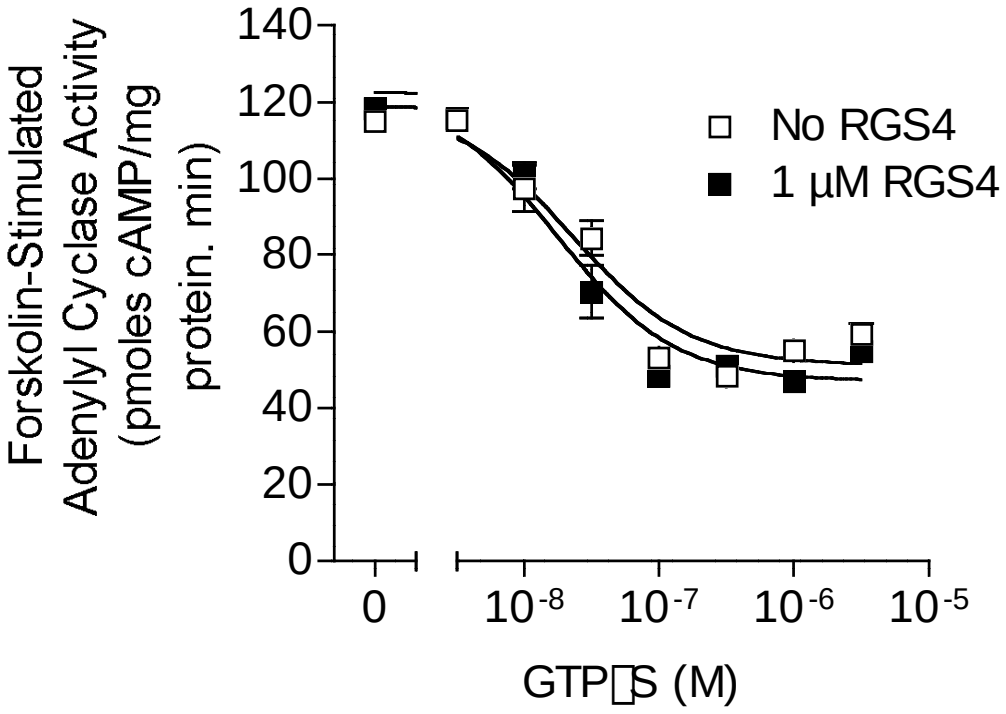
Forskolin-Stimulated
Adenylyl Cyclase Activity
(pmoles cAMP/mg
protein. min)
140
120
100
80
60
40
20
0
0
10-8
10-7
10-6
10-5
GTPγS (M)
No RGS4
1 μM RGS4

Figure 4.

Figure 5. RGS4 does not affect DAMGO-stimulated [^{35}S]GTPγS binding to SH-SY5Y membranes. Increasing concentrations of DAMGO were used to stimulate [^{35}S]GTPγS binding in SH-SY5Y cells. Binding in response to DAMGO did not differ significantly between the control (absence of RGS4) and treated (presence of 1μM RGS4) groups. DAMGO maximally stimulated [^{35}S]GTPγS binding 161 ± 5.5 % in the absence of RGS4 and 169 ± 6.5% in the presence of RGS4 ($t = 0.99$, $p = 0.3279$, not significant). The EC_{50} of DAMGO was 39 nM in the absence of RGS4 (95% confidence interval;15 to 96 nM) and 62 nM in the presence of RGS4 (95% confidence interval; 22 to 170 nM). In three independent experiments, DAMGO maximally stimulated [^{35}S]GTPγS binding 130 ± 7.7 and 100 ± 6.9 % above basal levels in the absence and presence of 1 μM RGS4, respectively ($t = 1.374$, $p = 0.1717$, not significant). The EC_{50} of DAMGO was 86 nM in the absence of RGS4 (95% confidence interval; 25 nM to 290 nM) and 55 nM in presence of RGS4 (95% confidence interval; 15 to 215 nM).

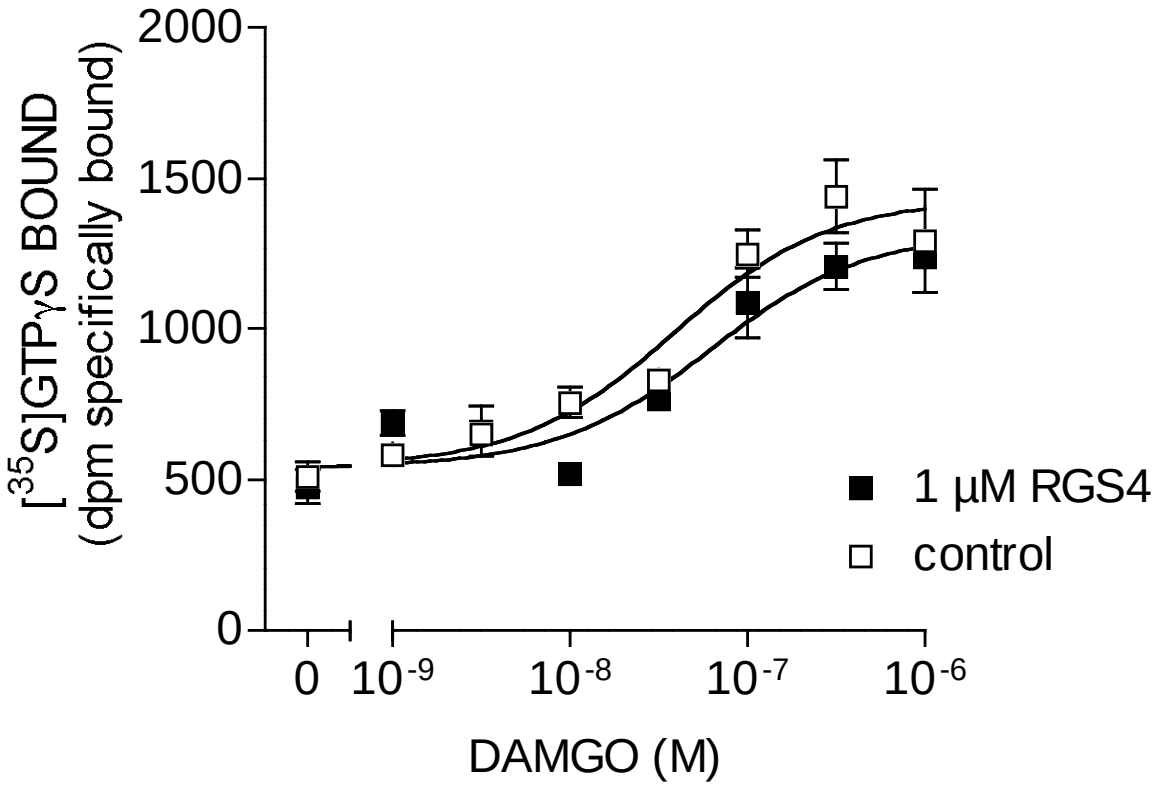
2000
1500
1000
500
0
[35S]GTPγS BOUND
(dpm specifically bound)
0
10-9
10-8
10-7
10-6
DAMGO (M)
1 µM RGS4
control

Figure 5

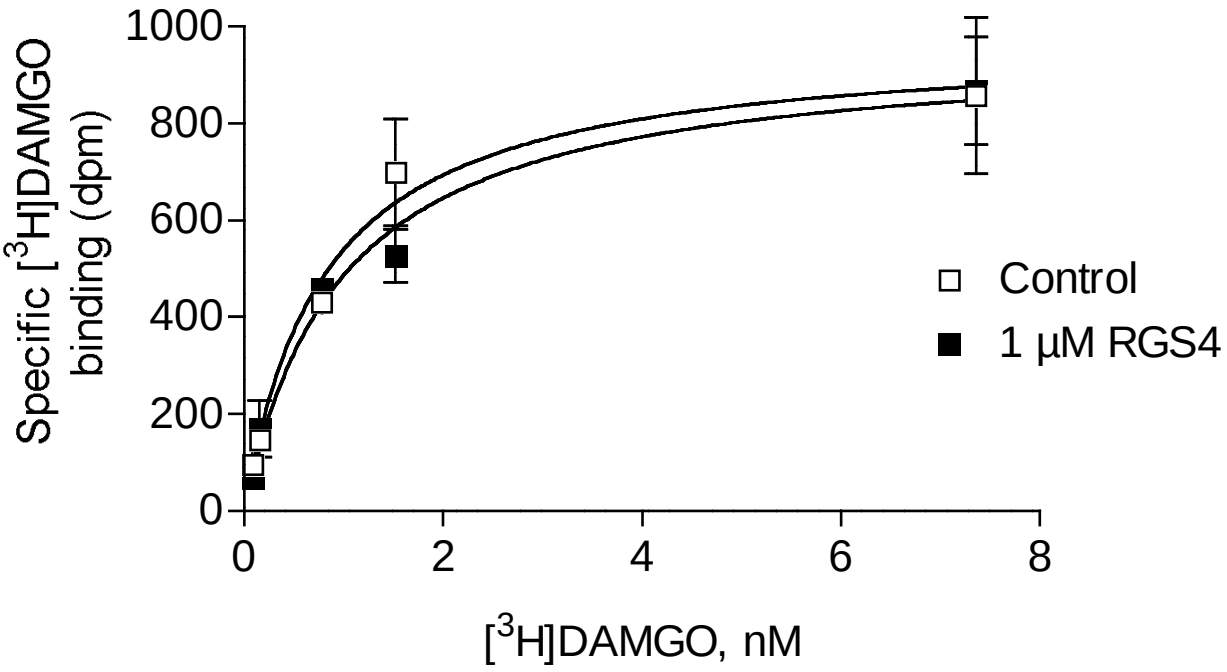

Figure 6. RGS4 does not affect [^{3}H]DAMGO binding to the mu opioid receptor on SH-SY5Y cell membranes. [^{3}H]DAMGO saturation binding was performed in the absence (open squares) or presence (filled squares) of 1 µM RGS4. RGS4 failed to affect either maximal [^{3}H]DAMGO binding or the K_D of [^{3}H]DAMGO. Data represent dpm ± SEM (n = 3) of specifically bound [^{3}H]DAMGO in a single experiment. In two independent experiments, the K_D values of [^{3}H]DAMGO were determined to be 0.80 and 0.97 in the absence and presence of RGS4, respectively; the B_{max} values of [^{3}H]DAMGO were 58.8 and 58.2 fmol/mg protein in the absence and presence of RGS4, respectively.

Chapter 3

Co-expression of the mu opioid receptor and RGS4 protein in regions of the rat brain:
Fentanyl treatment affects RGS4 sub-cellular location.

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Running title: Co-expression of mu opioid receptors and RGS4 in rat brain.

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SUMMARY

Opioid agonists exert their primary effects, including analgesia and reward through the inhibitory G protein-coupled mu opioid receptor. However, chronic opiate agonist administration results in the development of tolerance and thus decreased drug efficacy. **R**egulators of **G** protein **S**ignalling (RGS) proteins diminish signal transduction by enhancing the GTPase activity of the Gα subunit of the G protein. One of the RGS proteins, RGS4, may play a role in the development of desensitization of the mu opioid receptor. RGS4 mRNA is found throughout the brain, including regions that also express high levels of mu opioid receptors. Using antibodies generated against the C-terminal 50 amino acids of the rat mu opioid receptor and antibodies to the C-terminal 36 amino acids of rat RGS4, we were able to co-localize the two proteins in selected regions of the rat brain by immunohistochemical studies. The mu opioid receptor was found on the cell surface of neurons while RGS4 was found concentrated primarily in the nucleus of neurons. Changes in the subcellular localization of RGS4 were seen in rat brain sections from animals that had been treated with fentanyl, a potent mu opioid receptor agonist. In these sections, some of the immunoreactive RGS4 protein was observed to translocate from the nucleus to the cytosol. These results suggest that the administration of an opioid agonist results in a subcellular redistribution of RGS4 from the nucleus to the cytosol and the cell membrane where it may desensitize mu opioid receptor signalling via interaction with receptor-associated G proteins.

Key Words: Mu opioid receptor, RGS4, antibody, fentanyl, immunohistochemistry, locus coeruleus, habenula, hippocampus.

INTRODUCTION

For centuries morphine and related opioid drugs have been recognized for their actions as highly effective analgesics and thus have been the therapeutic choice for the relief of severe acute and chronic pain. Unfortunately, the clinical use of these drugs is limited by their tendency to cause desensitization of the mu opioid receptor following chronic use (1). The development of tolerance results in the need to use progressively larger doses of drug to achieve analgesia.

The mu opioid receptor is a member of the G protein-coupled receptor (GPCR) superfamily that transduce extracellular signals into cellular responses (2). Agonist binding to a GPCR induces a conformational change in the receptor from a low affinity to a high affinity agonist-binding state catalyzing the exchange of GDP for GTP on the receptor-associated G protein □ subunit. The activated G protein heterotrimer uncouples from the receptor and dissociates into GTP-□ and □□subunits which are then free to regulate effector systems. Signal termination is achieved by hydrolysis of □□GTP to □□GDP, via an intrinsic □-GTPase, and reassociation of the G protein heterotrimer. Mu opioid receptor-mediated activation of associated inhibitory G proteins results in hyperpolarization of the cells as a result of an increase in K^+ currents, an inhibition of voltage-dependent Ca^{2+} channels, and an inhibition of adenylyl cyclase activity (3-5).

Many cellular modifications to chronic agonist stimulation of the mu opioid receptor may contribute to the development of tolerance. The roles of GRK2-dependent phosphorylation, the recruitment of □ arrestin to the receptor, mu receptor internalization, and desensitization of G protein-gated, inwardly

rectifying K^+ channels (GIRKs) is desensitizaton are well established (6-9). Mu agonists differ widely in their abilities to induce these changes at the receptor level, suggesting that adaptations within the signal transduction pathway, downstream from the receptor itself may contribute to the development of this phenomenon (10).

Regulators of G protein Signalling proteins (RGS) represent a new class of proteins that negatively regulate GPCR-coupled signalling by acting as **G**uanosine triphosphatase-**A**ctivating **P**roteins (GAPs) to G proteins, greatly increasing the rate of GTP hydrolysis (1). The distribution pattern of RGS mRNA in the rat brain indicates that specific RGS subtypes could be associated with specific receptor signalling systems (11). RGS4 has been shown to increase the GTPase activity of Gi-types of G proteins, the same types of G proteins that mediate the downstream effects of the mu opioid receptor (12-16). Further, the chronic administration of morphine has been shown to regulate RGS4 mRNA levels in regions of the brain associated with anti-nociception and the development of tolerance and desensitization (17). The distribution of RGS4 in the brain has only been approximated by *in situ* hybridization studies because of the lack of good quality antibodies needed for a detailed immunohistochemical analysis of the regional and subcellular localization of the protein.

The current study was conducted to determine if RGS4 protein is expressed in the same neuronal populations that also express mu opioid receptors. Antibodies generated against rat RGS4 were used to localize RGS4 protein in discrete regions of the rat brain that express mu opioid receptors. We also

evaluated whether the administration of fentanyl, a mu opioid receptor-selective agonist, affects the sub-cellular distribution of RGS4. The results of this study lend further support for a role of RGS4 in the development of mu opioid receptor desensitization and suggest a novel mechanism by which high-dose opioid administration may contribute to the development of tolerance.

MATERIAL AND METHODS

Animals. Male Spraque-Dawley rats (Taconic, Germantown, NY, U.S.A.) weighing 200-225 g were used. Rats were housed in standard laboratory cages (2/cage) and kept in a temperature- and humidity-controlled colony room at least one week before treatment. Food and water were available *ad libitum.*

Fentanyl and saline treatment. Groups of three male Sprague-Dawley rats (200-225 g) were injected with fentanyl (56 µg/kg) or saline i.p. Two hours after injection, the rats were anesthetized and perfused transcardially with 4% formalin. The brains were subsequently removed and processed as described below.

Drugs, secondary antibodies, and mounting media. Alexa Fluor 555 and 645 goat anti□rabbit secondary antibodies, Vectashield, Vectashield plus DAPI, and Vectashield plus propidium iodide mounting medias were purchased from Molecular Probes (Eugene, OR). NeuN monoclonal antibody was obtained from Chemicon, Inc. (Temecula, CA).

Antibody preparation

Mu opioid receptor antibody preparation. Polyclonal antibodies were raised against a glutathione-S-transferase fusion protein that contained the C-terminal 50 amino acids of the rat mu opioid receptor (GST-$MOR_{349\text{-}398}$), and against a glutathione-S-transferase fusion protein that contained 61 amino acids from the N-

terminal region of the rat mu opioid receptor (GST-MOR_{10-70}), as previously described (18).

RGS4 antibody preparation. Polyclonal antibodies were raised in rabbits against a fusion protein consisting of glutathione-S-transferase (GST) and the C-terminal 36 amino acids of the rat RGS4 protein (GST-$RGS4_{170-205}$). PCR was used to generate DNA coding for the selected polypeptide sequence of rat RGS4 protein by reacting the primers shown below with rat RGS4 cDNA and the thermostable DNA polymerase *Pfu* (Stratagene) for 26 cycles on a Perkin Elmer Thermal Cycler using standard conditions. The following primers were used to generate cDNA coding for a protein composed of the 36 amino acid polypeptide that occurs at the C-terminal of the RGS4 protein:

5'-CGCG **GGA TCC** AAG TCT CGA TTC TAT CTT GAC CTG-3' (sense).

5'-GCGC <u>GAA TTC</u> TTA GGC ACA CTG AGG GAC TAG GGA-3' (antisense)

Our sense primer and antisense primer have *Bam*H1 and *Eco*R1 restriction sites at their 5' ends, indicated by bold print and underscore, respectively. A stop codon was placed in the antisense primer to ensure that the C-terminal of the fusion protein would be identical to that of the rat RGS4 protein. These primers resulted in the generation of a 128 base pair product in the PCR reaction.

The purified PCR product was digested with *Bam*HI and *Eco*RI endonucleases and ligated into linearized pGEX-2T as previously described (18). Competent JM109 *E. coli* bacteria (Promega; Madison, WI) were transformed with pGEX-2T

plasmids containing the PCR-generated DNA. The resulting plasmid was isolated from the transformants using the Quantum Prep Plasmid Midiprep Kit (BioRad) and then used as the template for automated fluorescent DNA sequencing in both the 5' and the 3' directions to ensure that no mutations had been introduced and that the sequence was in frame. The fusion protein was expressed in JM109 cells by the addition of 0.1 mM isopropyl □-D-thiogalactoside (IPTG). The fusion protein was extracted from the bacteria exactly as described by Levey (19) and purified on Glutathione-Sepharose 4B resin (Amersham Pharmacia). Glutathione was removed by Sephadex G-50 chromatography.

The GST-RGS4$_{170-205}$ fusion protein was used to immunize two rabbits. Antibodies were produced commercially (Duncroft Inc. VA) by injecting rabbits with fusion proteins using standard techniques. Initially, 200 µg of each fusion protein was emulsified in Freund's adjuvant and injected into multiple sites on the rabbit's back and subsequently, monthly injections of fusion protein were given in incomplete adjuvant. BLAST searches of Genbank indicated that the 36 amino acid polypeptide expressed at the C-terminal of the rat RGS4 protein shares no homology with any other protein sequences, including any of the other RGS protein family members. This 36 amino acid sequence differs by one amino acid (97% identity) from mouse RGS4, and differs from human RGS4 by two amino acids (94% identity). The next nearest sequence is *Xenopus* RGS4, which shares 17 of the 36 amino acids (47%). Thus, we feel that it is highly unlikely that antibodies raised against this fusion protein will cross-react with other RGS

proteins. Both the mu receptor antibody and the RGS4 antibody were antigen-affinity purified as previously described before being used in all experiments (18).

Tissue preparation for immunohistochemical staining. Male Sprague-Dawley rats (~225 g) were deeply anesthetized with sodium pentobarbital (i.p. 100 mg/kg). Rats were perfused transcardially with 0.5% sodium nitrite in 0.01 M phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4 (pH 7.4) at 25 C, followed by 4% neutral formalin in PBS. The brains were removed and post-fixed for 30 min in ice-cold 10% formalin, then transferred to 20% sucrose in PBS for 24 hours at 4 ºC. The brains were frozen in powdered dry ice, and 20 µm slices were obtained in a cryostat at -18 ºC and mounted onto twice coated chrom-alum gelatin slides as previously described (20). Slides were stored at -80 ºC until use for immunohistochemistry.

Immunohistochemistry**.** Fixed brain slices were incubated with rabbit anti-rat mu receptor antibody (1.0 µg/ml in 0.3% Triton-X 100 in PBS and 10% normal goat serum) or rabbit anti-rat RGS4 antibody (0.3 µg/ml in 0.3% Triton-X 100 in PBS and 10% normal goat serum) at 4 ºC for 48 h. Slides were washed twice for 10 min in 0.2% Triton X-100/PBS, and bound primary antibody was detected by the addition of Alexa Fluor 555 goat anti□rabbit secondary antibody (1:100).

Immunohistochemical controls included the preabsorption and coincubation of the mu receptor antibody with 0.1 µM of the mu receptor fusion protein against

which the antibody was raised (GST-$MOR_{349-398}$), and the preabsorption and coincubation of the RGS4 antibody with 0.1 μM of the RGS4 fusion protein against which the antibody was raised (GST-$RGS4_{170-205}$).

RESULTS

Identification of RGS4 and mu opioid receptors in rat brain. The current study was conducted to determine if RGS4 was present in selected brain regions that express mu opioid receptors. Adjacent rat brain sections were incubated with antigen-affinity purified antibodies raised against either the C-terminal 50 amino acids of the rat mu opioid receptor ($MOR_{349-398}$, 1.0 µg/ml) (18), or against the C-terminal 36 amino acids of rat RGS4 ($RGS4_{170-205}$, 0.3 µg/ml). RGS4 immunoreactivity was observed in the brain. Perusal of brain areas revealed 3 areas of interest that were influenced by an opioid agonist. We therefore have chosen to study the hippocampus, habenula and locus coeruleus, all of which contain a dense population of mu opioid receptors (21). A more complete study of RGS4 localization in the rat brain will be described in a later publication.

Mu opioid receptors and RGS4 were identified in the pyramidal cell layer (CA1-CA3) and dentate gyrus of the hippocampus (Figs. 1 A and B, respectively). The highest density of both proteins is seen in the pyramidal cell layers of CA1-CA3 and the granule cell layers of the dentate gyrus. Preabsorption of mu receptor antibodies with 0.1 µM GST-$MOR_{349-398}$ completely blocked the antibody recognition of mu opioid receptor (Fig. 1 C). Preabsorption of RGS4 antibodies with 0.1 µM GST-$RGS4_{170-205}$ completely blocked recognition of RGS4 protein (Fig. 1 D).

Consistent with the observations of Mansour (1995), mu receptor reactivity is more dense in the medial habenula than the lateral habenula (Fig. 2 A). RGS4 immunoreactivity is predominantly in the nuclei of the medial habenula, and

noticeably absent from processes (Fig. 2 B). Higher magnification reveals that the mu receptor appears to be expressed at the surface of the cell bodies and processes (Fig. 2 C). RGS4 appears to be primarily in the cell nuclei, with absence of immunoreactivity in the nucleoli and processes (Fig. D).

The locus coeruleus is a relatively homogenous neuronal population that expresses high levels of the mu opioid receptor (21). Additionally, RGS4 mRNA levels increase in this region following morphine treatment (17,22). Immunohistochemical staining of the locus coeruleus reveals the similar expression of both the mu opioid receptor and RGS4 (Fig. 3 A and B, respectively). As in the habenula, higher magnification shows the staining of the mu receptor on the surface of locus coeruleus cells and their neuronal processes, in contrast to the staining of RGS4 primarily in the cell nucleus (Figs. 3 C and D).

Subcellular localization of RGS4. Overlapping expression of mu opioid receptors and RGS4 was seen in the pyramidal cell layers of the hippocampus, granule cell layers of the dentate gyrus, the medial habenula, and the locus coeruleus (Figs. 1-3). Because higher magnification revealed that mu opioid receptors were expressed on the surface of neuronal cell bodies and fibers and RGS4 appeared to be in the nuclei of cells in these same regions, we were interested in confirming that RGS4 was localized in the nuclei of cells in untreated animals. Therefore, we double labelled sections using DAPI, a nuclear counterstain, and anti-RGS4$_{170\text{-}205}$. In the CA3 region of the hippocampus, the nuclei and nucleoli were clearly visible with DAPI (blue, Fig. 4 A). RGS4 is

present in many of the same regions (green, Fig. 4 B). Coexpression of DAPI staining and RGS4 protein was expressed by the color change, from blue (DAPI) or green (RGS4) to turquoise (Fig. 4 C). DAPI and RGS4 antibodies costained most large nuclei, but were not observed to colocalize in most small nuclei (likely glia; blue, panel C). In cells staining positive for DAPI and RGS4, the nucleoli remained blue due to the lack of RGS4 reactivity in this region. Because DAPI stains DNA in neuronal and non-neuronal cells, we examined neuronal populations with a neuronal specific marker, NeuN. Sections of CA3 were double-labelled with NeuN and with RGS4 antibodies. The neuronal cell bodies were revealed as red (Fig. 4 D) and the nuclei, containing RGS4, as green (Fig. 4 E). When the images were merged, coexpression of NeuN and RGS4 appeared yellow (Fig. 4 F). It appeared that all cells staining positive for RGS4 were also positive for NeuN, suggesting that RGS4 is expressed only in neurons.

Fentanyl treatment induces RGS4 translocation from nucleus. In order to attenuate mu receptor signalling, RGS4 would have to become associated with the mu receptor/G protein complex, or at least be associated with the activated G protein. To study this, fentanyl (a mu selective agonist) or saline was administered to rats to determine if mu receptor activation could affect the sub-cellular location of RGS4. Two hours post-injection (56 µg/kg), the rats were sacrificed by formalin perfusion, and the brains were prepared for immunohistochemical analysis as described in Materials and Methods. Coronal sections (20 µm) were screened for the presence of RGS4 with affinity-purified

rabbit anti-RGS4$_{170-205}$. Sections containing hippocampal pyramidal CA3 neurons from saline-treated rats were double labelled with DAPI and RGS4 antibodies (Fig. 5 A and C), and merging DAPI and anti-RGS4 images reveals that RGS4 remains primarily localized in the nucleus (Fig. 5 E.). In contrast, sections from fentanyl-treated rats containing the same region also double labelled with DAPI and RGS4 antibodies (Fig. 5 B and D) revealed that some RGS4 translocated from the nucleus to the cytosol (arrows, Fig. 5 D and E). Photos were taken at the most lateral aspect of the coronal slice of the rostral portion of the hippocampus.

Sections containing a region of the CA3 pyramidal cell layer were also compared in saline control and fentanyl treated rats using double labelling with NeuN and affinity-purified RGS4 antibodies (Figure 6). In sections from the control animal, RGS4 is found in the nucleus (Figs. 6 C and E), however, following fentanyl treatment, some RGS4 has translocated to the cytosol (arrows, Figs. 6 D and F).

Other regions were examined in sections from saline- and fentanyl-treated animals. When compared to sections from saline-treated animals, the fentanyl treatment appeared to elicit translocation of some RGS4 from the nucleus to the cytosol in the medial habenula (Fig. 7 A and B, respectively). Similarly, in the control animal, RGS4 is expressed most densely in the nuclei of neurons in the locus coeruleus (Fig. 7 C). The administration of fentanyl elicits translocation to the cytosol (Fig. 7 D).

Most interestingly, sections from all of the control animals revealed that cells of the nucleus of the mesencephalic tract (lateral to the locus coeruleus) expressed RGS4 in both the nucleus and the cytosol (Fig. 7 E). However, following fentanyl administration there was a dramatic increase in cytosolic RGS4 (Fig. 7 F). This neuronal population does express mu opioid receptors, though the role of opioids on this motor tract has not been extensively studied (23).

DISCUSSION

In the current study we have demonstrated the presence of RGS4 in discrete areas of the brain that also express mu receptors. To our knowledge, this is the first time that endogenous RGS4 has been identified in immunohistochemical studies. The amino acid sequence used to generate antibodies are unique to rat RGS4. They share 97% identity with mouse RGS4, 94% identity with human RGS4, and only 47% homology with *Xenopus* RGS4. Because the 36 amino acids share no homology with any other RGS proteins, we feel it is highly unlikely that our antibodies are cross-reacting with any other RGS protein.

A subset of RGS proteins have been shown to increase the GTPase activity of Gi-type G proteins, the type of G proteins that mediate the downstream effects of the mu opioid receptor (12-16). Of these, only RGS2 and RGS4 mRNA have been detected in most of the brain regions that express mu opioid receptors (11,21,24). However mRNA and/or immunoreactivity is absent in certain regions of the brain containing high levels of mu receptors and RGS4, notably the medial habenula and ventral tegmental area, while RGS4 mRNA is present in these regions (24). Additionally, RGS4 protein demonstrates clear specificity for Gαi subfamilies, in contrast to RGS2, which preferentially acts on Gαq (16,25).

Our laboratory has determined that the addition of recombinant RGS4 to SH-SY5Y cell homogenates caused a concentration-dependent attenuation of mu opioid receptor-mediated inhibition of adenylyl cyclase activity (A. T. Crowder, D. Jacobowitz, H. Weems, T. Cote, unpublished observations). Additionally, Nakagawa, *et al.* (2001) demonstrated agonist activation of the mu opioid

receptor in the rat pheocytoma PC12 line resulted in an up-regulation of RGS4 mRNA. The time course of the up-regulation paralleled the time course profile of mu opioid receptor desensitization. Further evidence for a functional connection between RGS4 and mu opioid receptor signalling has come from overexpression of RGS4 in HEK293 cells stably expressing the mu opioid receptor. In this system, RGS4 attenuated agonist-mediated inhibition of adenylyl cyclase activity (26). While it is quite possible that one or more of the other RGS proteins are also involved in mu opioid receptor signalling, the distribution and functional characteristics of RGS4 make it one of the most likely of the RGS proteins to be involved in mu opioid receptor desensitization.

We demonstrate the expression of both the mu opioid receptor and RGS4 in selected regions of rat brain (hippocampus, habenula, and locus coeruleus). Other studies have reported the development of antibodies to RGS4 that recognize the protein in a Western blot analysis, but are unsuitable for immunohistochemistry (17). RGS4 was identified within selected regions of rat brain using affinity-purified antibodies directed against the C-terminal 36 amino acids of rat RGS4 ($RGS4_{170-205}$) to immunohistochemically screen brain sections. The location of RSG4 protein was consistent, overall, with the previously reported location of RGS4 mRNA (11). We are currently mapping the complete localization of RGS4 protein throughout the rat brain.

Two previously developed mu opioid receptor antibodies, one against the C-terminal region of the rat mu receptor and one against the N-terminal region of the rat mu receptor, were used to immunohistochemically detect mu opioid

receptors in rat brain. We have shown that these antibodies specifically immunoprecipitate soluble mu opioid receptors, and the C-terminal directed antibody recognizes endogenous mu opioid receptors in Western blots following their immunoprecipitation from rat brain membranes (18). In these immunohistochemical studies both of these mu receptor antibodies labelled the exact same structures, and were in the same regions previously reported by Mansour to contain mu opioid receptors (21). The regions included the neocortex, hippocampus, locus coeruleus, periaquaductal grey, medial habenula, thalamic and hypothalamic nuclei, and two major neuronal pathways, the fasciculus retroflexus and the stria terminalis (data not shown) (21). Additionally, presorption with each antigen, at a concentration of 0.1 μM, specifically blocked the ability of the appropriate antibody to identify the mu opioid receptor. Since both mu receptor antibodies and RGS4 antibodies were raised in the same species, we were unable to perform double labelling experiments on the same brain sections. However, when adjacent sections were individually labelled with each antibody, it appeared that the two proteins were co-expressed in many of the same types of neurons in the same regions.

Region-specific expression of RGS mRNA has been demonstrated in rat brain and regional expression of RGS protein is one likely mechanism of specificity between RGS proteins and G-protein □ subunits (11). Specificity may also arise at the molecular level where specific RGS protein recruitment is dependent upon specific activation of a particular G□ subfamily. Differential regulation, however, requires physiological signals that provide state-dependent specificity (25). For

RGS4 to be able to regulate mu receptor signalling, it would have to be localized in neurons that express mu receptors, as well as be able to associate with the receptor and/or its associated G proteins and effectors. In untreated animals, immunohistochemical staining revealed that RGS4 is primarily localized to the nucleus of neurons. RGS4 contains both a nuclear import and export sequence suggesting that its transport into and out of the nucleus may be regulated (27). Chattergee and Fischer (2000) reported that in COS7 cells, transfected with GFP-tagged human RGS4, the RGS4 protein accumulated in the cytoplasm. The protein was found in the nucleus only following deletion or mutation in the nuclear export sequence (27). However, in agreement with our findings, other RGS proteins, including RGS2, RGS8, and RGS10, have been shown to accumulate in the nucleus (28-30). Since the development of tolerance occurs as a response to chronic receptor stimulation, under normal conditions one would not expect to find high levels of RGS proteins in association with their receptors where they would interfere with normal receptor functioning.

We have demonstrated that endogenous rat RGS4 is found primarily in the nuclei of neurons in the pyramidal and granule cell layers of the hippocampus and dentate gyrus, the medial habenula and locus coeruleus. Two hours following fentanyl administration, RGS4 translocated from the nucleus to cytosol in the CA3 pyramidal cells of the hippocampus, in the medial habenula, and in the locus coeruleus. In the nucleus of the mesencephalic tract, it was determined that RGS4 is not localized only to the nucleus, but also in the cytosol. However, the administration of fentanyl resulted in a dramatic increase in cytosolic RGS4

protein. Opioid administration may result in a reversible modification at the N-terminal of endogenous RGS4 (possibly at the leucine-rich export sequence) that induces translocation from the nucleus, or it may cause a reversible modification of the import sequence that prevents its accumulation in the nucleus. We believe that chronic agonist binding to the mu receptor, along with some additional event, results in cellular signalling mediates translocation of RGS4 to the plasma membrane where it can interact with mu opioid receptor-associated G proteins and effectors.

There is evidence for the compartmentalization of signalling molecules, such as G proteins and adenylyl cyclase, in specialized regions of the plasma membranes termed lipid rafts that contain high levels of caveolin and protein acyltransferase activity (31-33). Li *et al* (1995) has reported the direct interaction of G proteins and caveolin (34). Recently, it was demonstrated that the palmitoylation of RGS4 Cys-2 and Cys-12 residues targets the protein to the lipid rafts where a subsequent palmitoylation of Cys-95 results in greatly enhanced GTPase activity (35,36). The observed cytosolic punctate pattern of RGS4 staining is consistent with a concentration of protein at discrete regions that perhaps includes the plasma membrane. Double immunofluoresence studies using antibodies to a protein known to associate with lipid rafts, such as caveolin, and RGS4 antibodies may enable us to determine if translocated RGS4 is concentrated in these regions of the plasma membrane (32).

RGS4 is found in some of the same regions of the brain that express high levels of mu opioid receptor. We have shown that the treatment of rats with mu

agonist results in a change in the subcellular localization of RGS4. These findings support the proposal that RGS4 may play a role in the desensitization of mu opioid receptor signalling in the rat brain.

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Figure 1. Overlapping expression of endogenous mu opioid receptor and endogenous RGS4 immunoreactivity in the rat hippocampus.

Immunostaining for the mu opioid receptor (left panels) and RGS4 protein (right panels) in coronal sections of rat brain. **(A)** Mu opioid receptor was detected in the pyramidal cell layers of the rat hippocampus and dentate gyrus with affinity purified antibodies (0.3 µg/ml) against the C-terminal 50 amino acids of the rat mu opioid receptor ($MOR_{390-398}$). **(B)** A similar pattern of expression was observed using antibodies directed against the C-terminal 36 amino acids of rat RGS4 ($RGS4_{170-205}$). Immunoreactivity was greatest in the pyramidal cell layers and granule cell layers, while decreased reactivity was observed in the dentate hilus. In panel **C**, pre-absorption of mu receptor antibody with 10^{-7} M GST-$MOR_{349-398}$ blocks detection of immunofluorescence. In Panel **D**, pre-absorption of RGS4 antibody with 10^{-7} M GST-$RGS4_{170-20}$ blocks immunofluorescence. Scale bar = 100 µm.

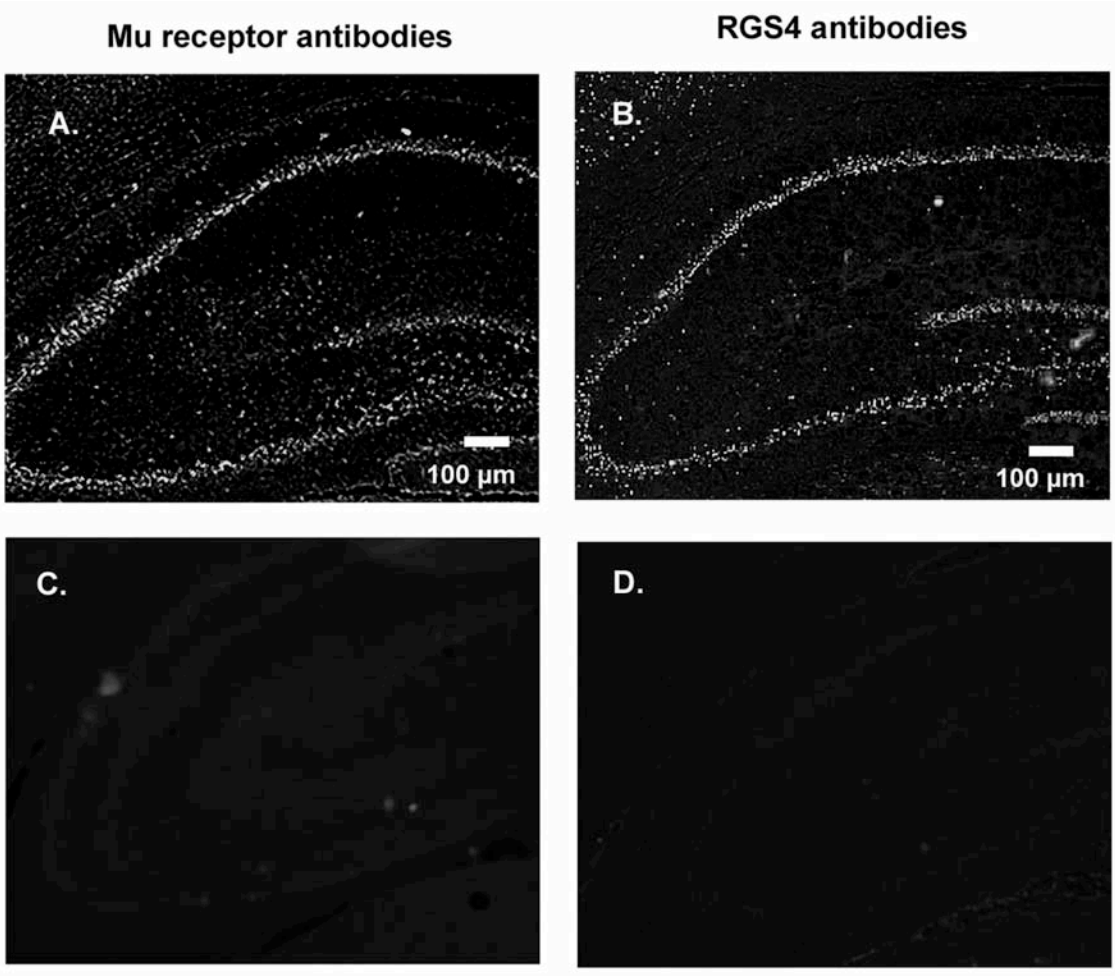


Figure 1

Figure 2. Overlapping expression of mu opioid receptor and RGS4 in the habenular nucleus. Antibodies against **(A)** mu receptor and **(B)** RGS4 both shared increased expression in the medial habenula compared to the lateral habenula (4X, scale bar = 50 µm). While RGS4 is expressed only in cell bodies, mu opioid receptors are also expressed in nerve processes. At a higher magnification (40X, scale bar = 10 µm), mu opioid receptors appear to be expressed at the cell surface and on neuronal processes of the medial habenular nucleus **(C)**. In contrast, RGS4 protein is expressed almost exclusively in the nuclei of medial and lateral habenular neurons **(D)**.

Mu receptor antibodies **RGS4 anitbodies**

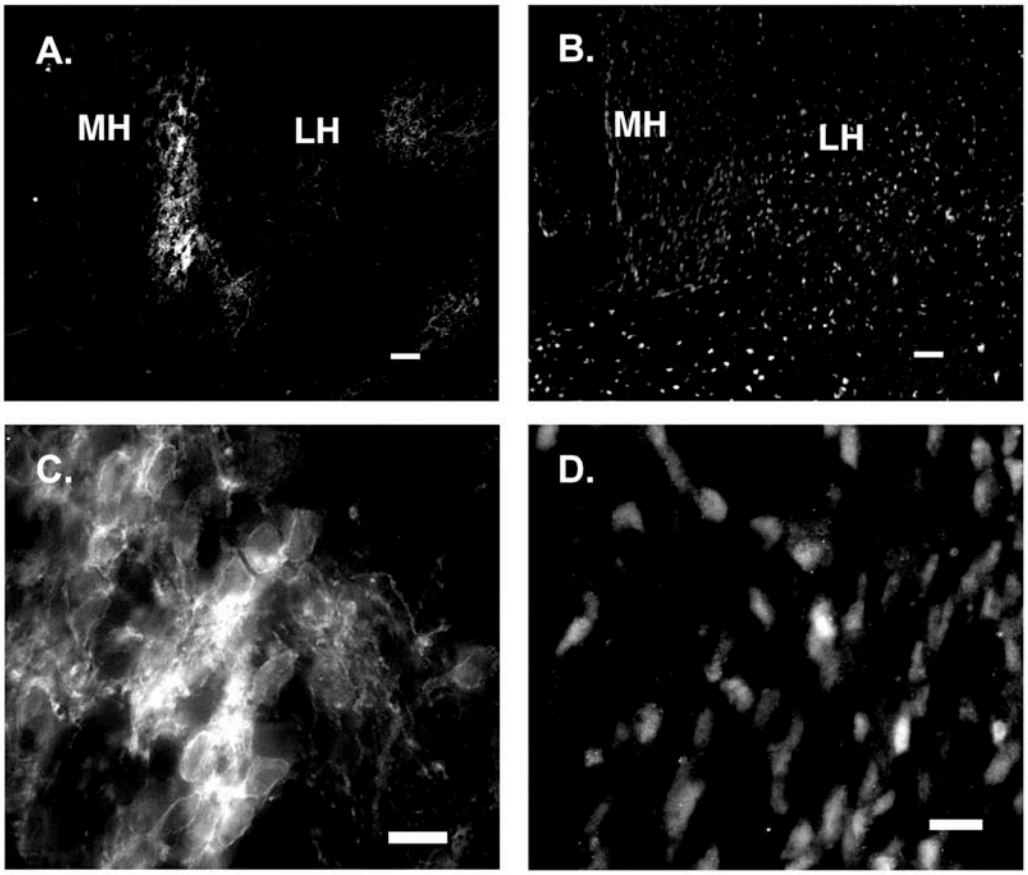


Figure 2

Figure 3. Overlapping expression of mu opioid receptor and RGS4 in the locus coeruleus. Mu opioid receptor **(A)** and RGS4 **(B)** immunoreactivity is seen in the locus coeruleus (4X scale bar = 100 µm). As in the habenula, at higher magnification (40X, scale bar = 10 µm), the mu opioid receptors **(C)** appear to be expressed at the cell surface and on neuronal processes while RGS4 protein **(D)** in the untreated animal is expressed almost exclusively in the nuclei.

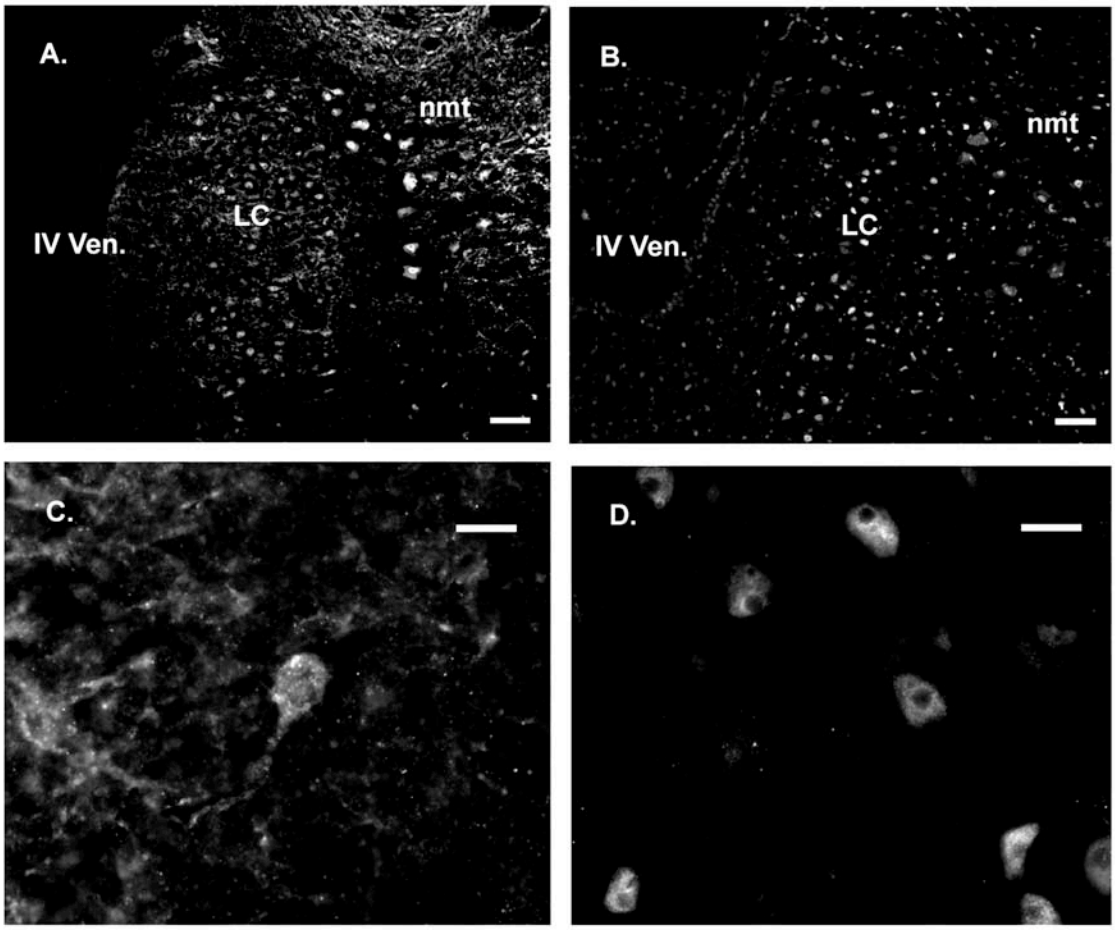


Figure 3

Figure 4. RGS4 is expressed in the CA3 pyramidal neurons. Left panels, DAPI labelling **(A)**, and RGS4 labelling **(B)**, in CA3 regions of rat hippocampus reveals RGS4 protein localized almost exclusively to cell nuclei in rat brain. When the images in A and B were merged **(C)**, overlapping staining appeared turquoise **(arrows, panel C)**. **Right panels.** An adjacent section through CA3 was stained for NeuN expression **(D)** and RGS4 **(E)**. Merging images in D and E resulted in yellow where expression of NeuN and RGS4 proteins overlap **(arrows, panel F)**. All cells positive for RGS4 were also positive for NeuN, suggesting that RGS4 expression may be limited to neurons. Image represents section from one rat, similar staining was seen in sections from all 3 animals. Scale bar = 10 µm.

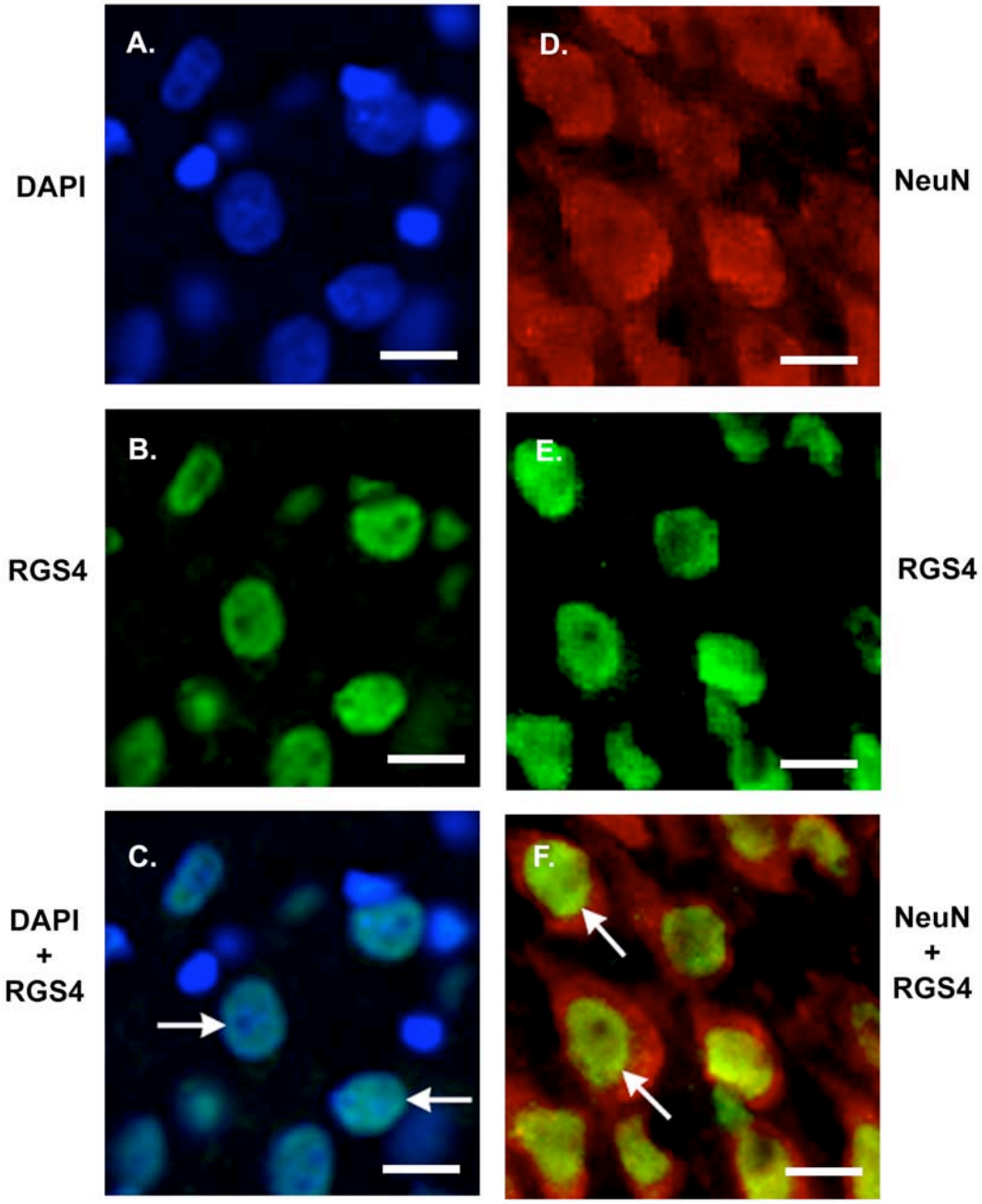


Figure 4

Figure 5. Fentanyl treatment caused the translocation of RGS4 from the nucleus to the cytosol of cells in CA3 regions of the hippocampus. Two hours after rats were injected with fentanyl (0.056 mg/kg), RGS4 expression was detected in cytosol of cells in CA3 layers. Merged images **(E)** from a section from a saline control rat, double labelled with DAPI **(A)** and RGS4 antibodies **(C)** revealed RGS4 expression primarily localized to the nucleus. In a section from a fentanyl treated rat, there is increased cytoplasmic RGS4 expression related to controls **(Figs 5 B, arrows D, and F)**. Images represent one saline treated and one fentanyl treated rat. Similar staining was seen in all 3 animals from each group. Scale bar = 10 μm.

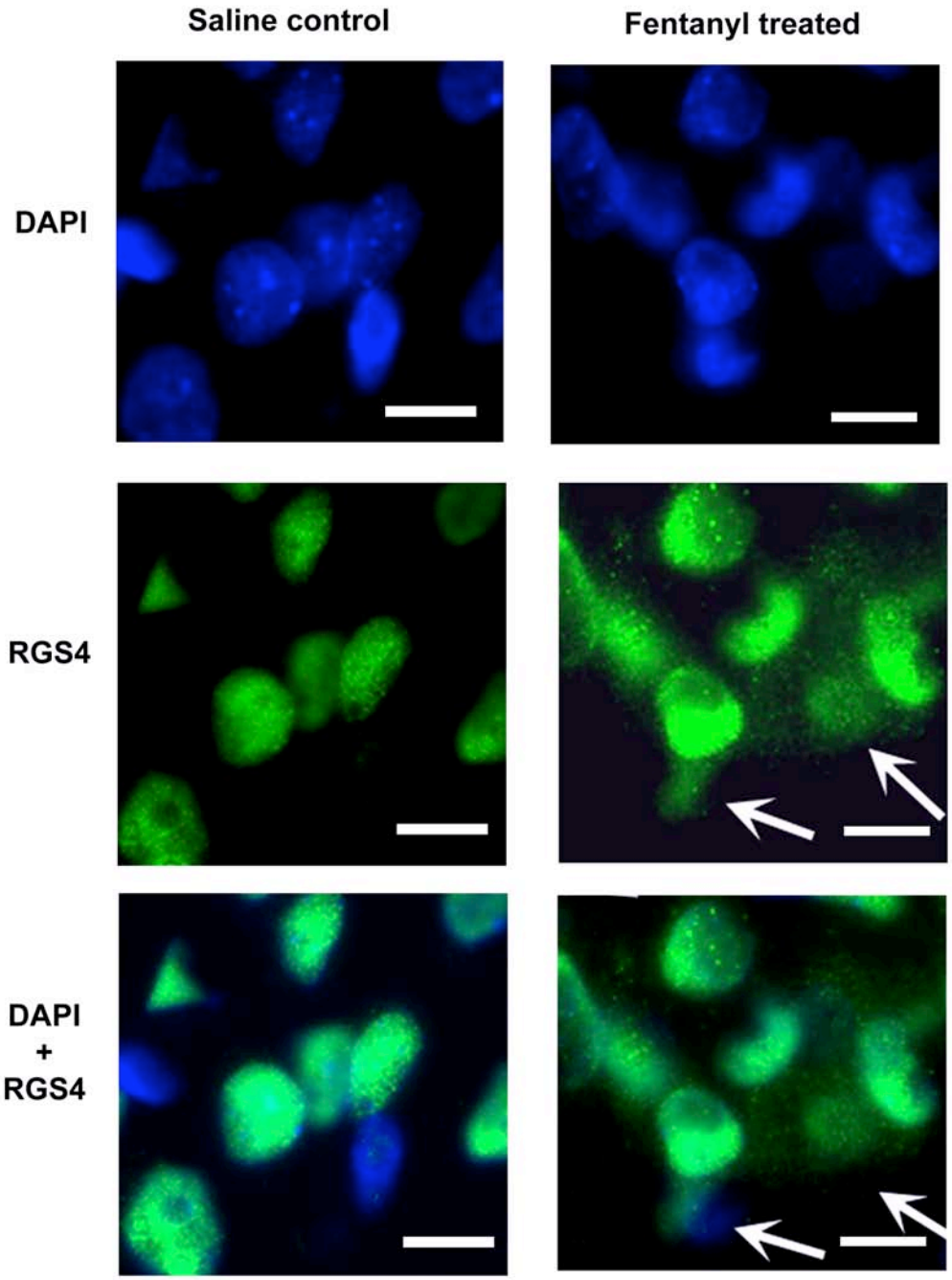


Figure 5

Figure 6. Fentanyl treatment caused the translocation of RGS4 from the nucleus to the cytosol of neurons of CA3 region of hippocampus. Sections from saline control and fentanyl treated rats were double labelled with a specific neuronal antibody, NeuN (red), and RGS4 antibodies (green). RGS4 is expressed in the nuclei of neurons in the control animal section **(Figs 6 A, C, and E)**. Section from a fentanyl treated animal reveal RGS4 translocation from the nucleus to the cytosol **(Figs 6 B, D, E)**. Scale bars = 10 μm.

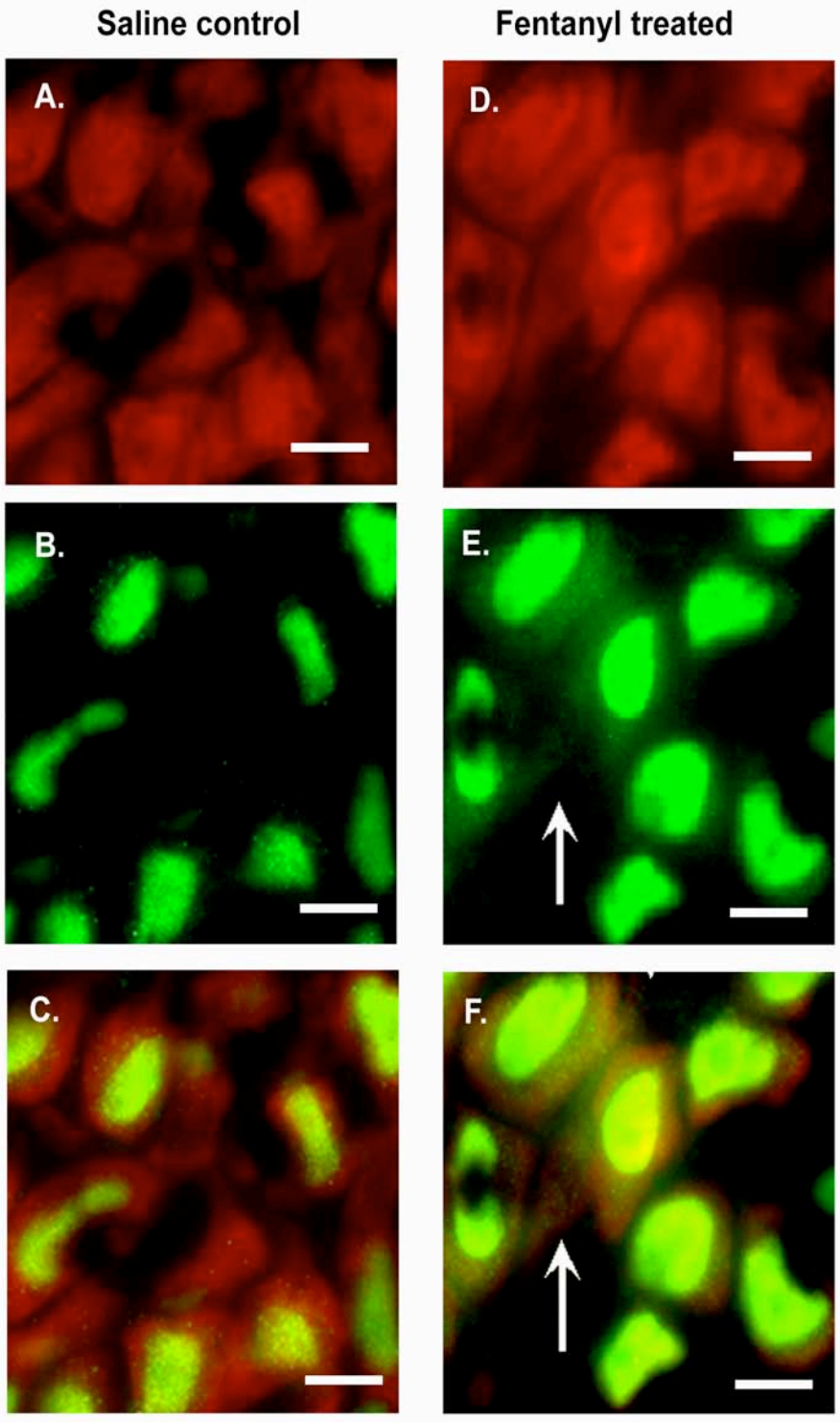


Figure 6

Figure 7. Fentanyl treatment caused the translocation of RGS4 from the nucleus to the cytosol of neurons of the medial habenula, locus coeruleus and nucleus of the mesencephalic tract. Double labelling, using propidium iodide DNA counterstain and RGS4 antibodies reveal that RGS4 is primarily in the nucleus of rat brain sections of medial habenula and locus coeruleus from saline-treated control rats **(A and C)**. In control sections, RGS4 is found in both the nucleus and cytosol in neurons of the nucleus of the mesencephlic tract **(E)**. Two hours after fentanyl treatment, RGS4 has translocated to the cytosol in the medial habenula and locus coeruleus **(arrows, B and D)**. In a representative section from a saline control rat, RGS4 is densely expressed in the nucleus of the mesenchephalic tract, with some protein seen in the cytosol **(E)**. Fentanyl treatment elicited a dramatic increase in cytosolic RGS4 in the neurons of this region **(F)**. Scale bars = 10 µm.

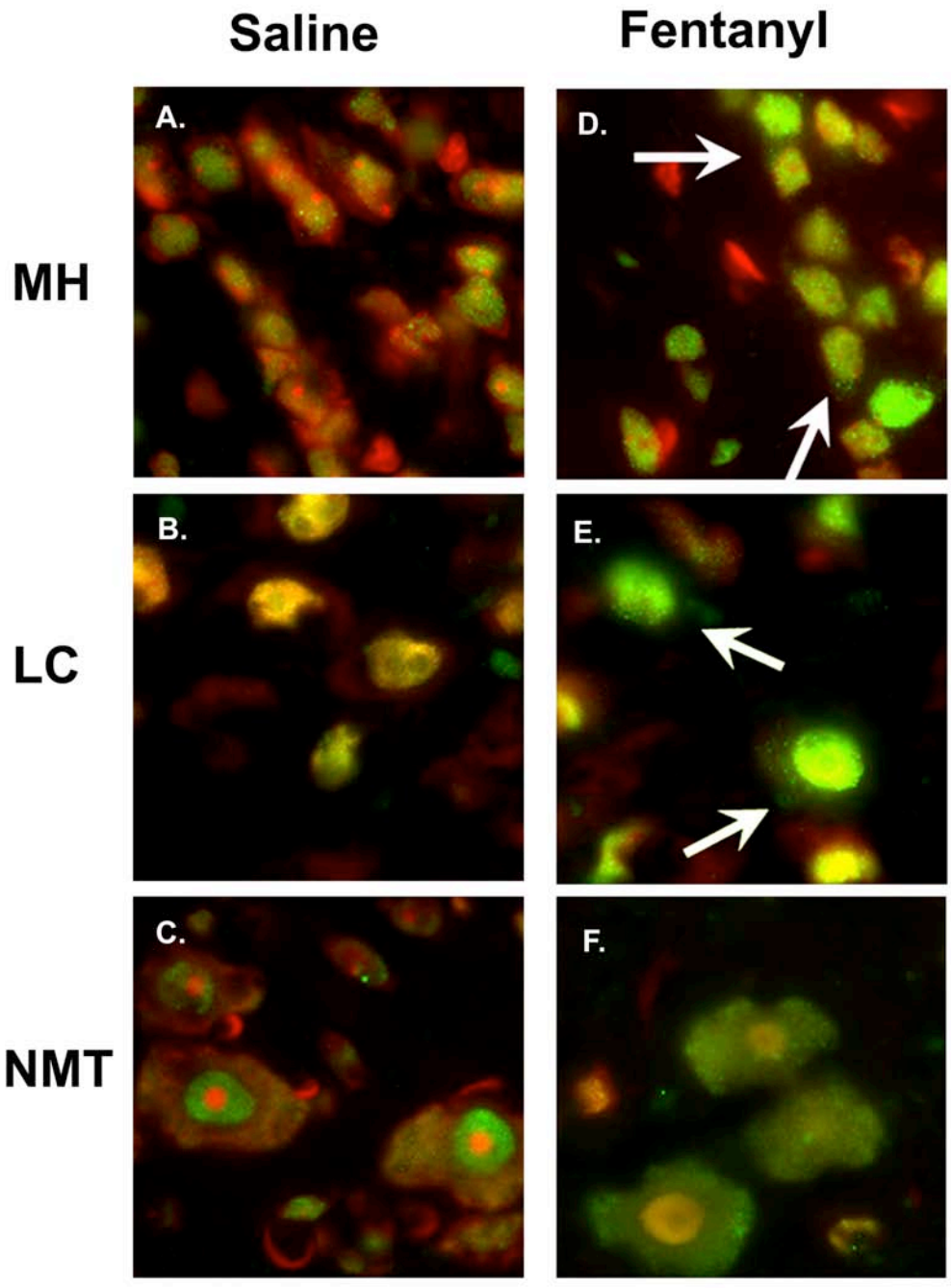


Figure 7

Chapter 4

Discussion and Future Directions

Opioid receptors interact with endogenous peptides to modulate pain pathways, mood, and reward. The mu opioid receptor is particularly important because it plays an important role in the management of nociception and is the primary target of endogenous peptide ligands, such as endorphin, and exogenous opioid drugs, such as morphine and fentanyl. Mu agonists remain the most effective drugs for the management of severe pain. Their clinical use is hampered, however, because sustained mu opioid receptor activation also produces undesirable cellular responses including the development of tolerance and dependence. Endogenous ligands, in contrast, activate the same receptors but do not induce physiological tolerance or dependence. Agonist binding to the mu opioid receptor activates its inhibitory Gi/Go, pertussis toxin-sensitive G proteins which leads to regulation of intracellular effectors, including inhibition of adenylyl cyclase activity, closure of voltage-gated Ca^{2+} channels, and activation of inwardly rectifying K^{+} channels (Chen et al., 1993; Chen and Yu, 1994; Moises et al., 1994). Though the basic cellular mechanisms of opioid receptor function are well characterized, the molecular mechanisms involved in the development of mu opioid tolerance following persistent agonist stimulation are complex and remain incompletely understood.

Several mechanisms have been proposed to explain desensitization, all of which involve intracellular adaptations to the effector changes. Adaptational changes directly affecting the receptor involve phosphorylation by G protein receptor kinases (GRKs) which reduce the ability of the receptor to bind G proteins, but not enough to sufficiently explain desensitization. GRK phosphorylation stimulates the subsequent binding of β-arrestin and dynamin 2 proteins, uncoupling of the receptor from its cognate G proteins, and internalization of the receptor/β-arrestin complex (Patel et al., 2002). Accordingly, uncoupling the receptor from its G proteins and receptor internalization clearly play a role in desensitization of mu opioid receptor signalling by reducing the number of G proteins to activate effectors and reducing the number of receptors available at the plasma membrane. Opioids with higher affinity for the mu receptor more effectively trigger the internalization process than opioids with lower affinity (Keith et al., 1998). Interestingly, morphine presents an unusual case among opioid agonists. Chronic morphine administration results in the development of tolerance. However, even though morphine increases mu opioid receptor phosphorylation, it only weakly activates GRK2 and causes neither an increase in receptor internalization nor a decrease in receptor number even when administered at doses much higher that required for maximal analgesia (Keith et al., 1996; Whistler et al., 1999). Thus, chronic morphine treatment brings about changes in neurons expressing mu receptors that impact mu receptor signal transduction without affecting mu receptor number or agonist binding. Differences in mu receptor internalization by agonists could

be a result of differences in receptor/agonist conformation that increase or decrease the ability to bind trafficking proteins. It is also possible that different agonists affect the levels of trafficking proteins directly. Etorphine, a powerful mu agonist, increases dynamin 2 protein levels by ~70%, whereas morphine has no effect on dynamin abundance (Patel et al., 2002). Clearly, the processes underlying the development of tolerance are intricate and involve multiple signalling processes.

In 1975, Cox identified two components of desensitization following chronic exposure to opioids (Cox et al., 1975). The first component was dependent upon the presence of the agonist and decayed rapidly upon removal of the drug. The second component was independent of the continued presence of the drug, and the effects were sustained for hours to days following agonist removal. This process is similar to the two-phase response to drug withdrawal in neurons of the locus ceruleus. The first, rapidly decaying response is probably the result of receptor uncoupling and internalization. The second response is slowly decaying and marked by sustained changes in G proteins and in increased adenylyl cyclase activity (Nestler, 1993; Lane-Ladd et al., 1997). Increased adenylyl cyclase activity, in the continued presence of agonist, is a widely accepted model for the development of tolerance.

Uncoupling of G proteins from their receptor and receptor internalization are examples of mechanisms that clearly result in tolerance. However, the development of tolerance in the absence of internalization, must involve additional components of the G protein coupled receptor signalling system. RGS proteins

counter the activity of G protein-coupled receptor signalling systems, thus functioning as negative regulators of G proteins. A consequence of negative regulation of G proteins is G protein-coupled receptor desensitization. Thus, the RGS family of proteins is an excellent candidate for contributing to the development of receptor-mediated desensitization. We chose to investigate the role of a particular member of the RGS family, RGS4, in the development of tolerance of the mu opioid receptor. RGS4 interacts with inhibitory G proteins, like those associated with the mu receptor, by enhancing the GTPase activity of the Gα subunit.

We demonstrated that the addition of recombinant RGS4 to homogenates from SH-SY5Y cells (a cell line expressing endogenous mu opioid receptors) resulted in a blunting of the ability of DAMGO, a potent mu agonist, to inhibit adenylyl cyclase activity in a concentration dependent manner (Chapter 2). The ability of DAMGO to inhibit adenylyl cyclase activity was reduced nearly 50%, from 55% inhibition to ~ 25%, in the presence of 3 µM RGS4. Recombinant RGS4 diminished the efficacy, but not the potency, of DAMGO in inhibiting adenylyl cyclase activity in SH-SY5Y cell homogenates. The potency of RGS4 in diminishing mu receptor was similar to the reported potency of RGS4 that increases the GTPase activity of Gi-type G proteins (Berman et al., 1996). Additionally, RGS4 had no effect on the ability of GTPγS to inhibit adenylyl cyclase activity or on the ability of a mu agonist to enhance [^{35}S]GTPγS binding to membrane-associated G proteins. These findings are consistent with the notion that RGS4 can diminish mu receptor signalling by inactivating the G-proteins

associated with the mu opioid receptor. Most importantly, the addition of RGS4 did not cause a reduction in receptor binding sites. This finding is consistent with its potential role in desensitization as occurs in the presence of prolonged stimulation by morphine or other agonists which do not induce receptor internalization.

If RGS4 plays a role in desensitization of the mu opioid receptor, then it must be present in regions of the brain that also express the receptor. Immuno-histochemical staining of rat brain sections, using antibodies against rat RGS4 and the rat mu opioid receptor, revealed that RGS4 protein and the mu opioid receptor are both expressed in the habenular nucleus and the locus ceruleus, regions of the brain involved in the development of tolerance and desensitization (Gold SJ, 2003), as well as the hippocampus, dentate gyrus and nucleus of the mesencephalic tract. These regions also corresponded to those previously reported for mu receptor and RGS4 mRNA localization (Mansour et al., 1995; Gold et al., 1997). We observed that RGS4 protein was localized primarily to the nucleus of cells, whereas the mu opioid receptors were on the plasma membrane of cells. To mediate mu receptor signalling, the RGS proteins must come into contact with the plasma membrane where they can interact with the receptor and/or its effectors. The N-terminal region of RGS4 has been implicated as important in the discrimination of receptor complexes coupled to Gq (Zeng et al., 1998). Additionally, the N-terminal 13 amino acid residues of RGS4 contain a leucine-rich nuclear export sequence, MCKGLAGLPASCL, that suggests it may be transported out of the nucleus.

One would not expect to find a protein that functions as a negative regulator of receptor signalling to be associated with the receptor under normal physiological conditions. The protein must be sequestered from the receptor so as not to attenuate normal receptor signalling. Because desensitization occurs with chronic agonist stimulation, we hypothesized that RGS4 might be in close proximity to the receptor and its G proteins only following agonist treatment. We compared the subcellular location of RGS4 in distinct rat brain regions in sections from control rats and rats treated for 2 h with fentanyl, a potent mu receptor agonist. In sections from fentanyl-treated animals, RGS4 translocated out of the nucleus and into the cytosol in neurons of the CA3 pyramidal layer of the hippocampus, the medial habenula, the locus ceruleus and the nucleus of the mesencephalic tract. In the control animals RGS4 was sequestered in the nucleus in all these regions with the exception of the mesencephalic tract nucleus. In this region RGS4 was found in the both the nucleus and cytosol in all the control animals. However, there was a marked increase of RGS4 in the cytosol following the fentanyl treatment.

The locus ceruleus is the principal noradrenergic nucleus in the central nervous system and has been extensively studied with regard to chronic opioid stimulation. Acute agonist stimulation results in inhibition of the activity of these neurons, but chronic agonist treatment results in the development of tolerance to inhibition and dependence upon the drug (Christie MJ, 1987). *In situ* studies revealed that RGS4 mRNA levels increased 2-3 fold 6 h following precipitation of opiate withdrawal in the locus ceruleus of rats treated for 6 days with morphine.

RGS4 protein levels in response to the morphine treatment were strikingly different, increasing 2-fold following morphine treatment, and decreasing to control levels within 6 h of precipitation of withdrawal (Gold SJ, 2003). These data suggest that a longer course of treatment is warranted to further characterize RGS4 translocation in the locus ceruleus and other regions of the brain. Analysis of RGS4 translocation, following fentanyl treatment for various time points, in other brain regions important in the analgesic and euphoric actions of opioid administration, including the thalamus and periaquaductal grey, the caudate putamen, nucleus accumbens, ventral tegmental area, and locus ceruleus, is necessary.

RGS4 is a good candidate for the desensitization of the mu opioid receptor. It is co-expressed in many of the same regions of the brain as the mu opioid receptor, is a GTPase activating protein for the inhibitory G proteins associated with the receptor, and both its mRNA levels and the subcellular location of its protein are affected by chronic mu agonist stimulation. We have found that the mu opioid receptor and RGS4 are localized in the hippocampus, the dentate gyrus, the medial habenula, the locus ceruleus, and the nucleus of the mesencephalic tract. Further, we have demonstrated that there is a functional interaction between mu opioid receptor signalling and RGS4.

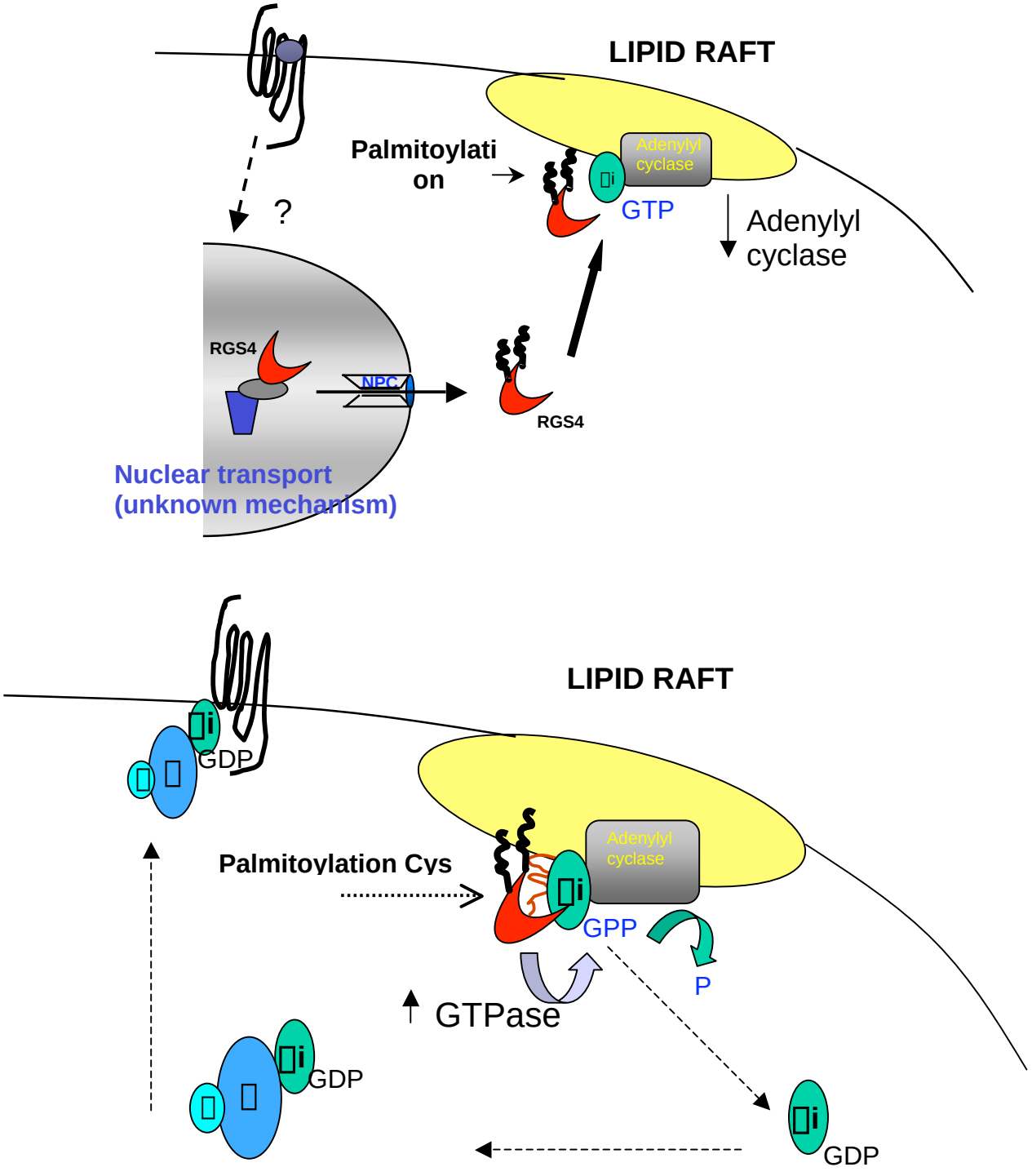
Possible Mechanisms of Action of RGS4 regulation of signalling

Previous site-directed mutagenesis studies of the mu opioid receptor have shown that G protein binding and activation occurs at the third intracellular loop of

the mu opioid receptor and does not involve the first 14 or the terminal 45 residues of the carboxyl tail (Georgoussi Z, 1997; Wang, 1999). The biological action of RGS4 protein is thought to depend on its ability to act as a GTPase-activating protein (GAP) (Berman et al., 1996). Analysis of the crystal structure of RGS4 and G□i revealed that the RGS box forms a four-helix bundle contacting the switch domains I, II, III of the G□ subunit (Tesmer et al., 1997). These switch regions are critical residues associated with the binding and hydrolysis of GTP. It is clear that RGS4 interacts with the G□ protein subunit. It is not clear whether or not RGS4 physically interacts with the mu opioid receptor. The inability of our antibodies, directed against the N-terminal residues of the receptor, to co-immunoprecipitate RGS4 suggests that RGS4 may not bind the mu receptor, or that the binding is too short lived to capture in the period of time required to solubilize and immunoprecipitate the mu receptor. The inability of antibodies, directed against the C-terminal residues of the mu receptor, to co-immunoprecipitate RGS4 also suggests that RGS4 may not bind the mu receptor. Crystallography studies have shown that RGS4 binds the G□ subunit only when the subunit is in its high-affinity state, a state in which it is uncoupled from its receptor.

If RGS4 does not bind the receptor, then it may bind the G□ subunit at an effector, such as adenylyl cyclase (Figure 1). Recently, RGS4 has been demonstrated to associate with lipid rafts regions of the plasma membrane (Osterhout JL, 2003). These regions contain dense populations of signal transduction molecules, including G□ subunits and adenylyl cyclase (Hiol A, 2003). The RGS4

Figure 1. Model of role of RGS4 in the desensitization of the mu opioid receptor. Agonist binding induces RGS4 translocation from the nucleus, to lipid raft regions by an unknown signal. Activated G subunits also move to lipid rafts to mediate effector pathways. RGS4 palmitoylation of Cys 2 and 12 occurs at membrane and enhances interaction with the lipid raft, where additional palmitoylation of Cys 95 occurs, enhancing contact with G subunits. In this position RGS4 increases the GTPase activity of the G protein, attenuating effector signalling.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protein that had been palmitoylated at residues Cys 2 and Cys12 had an increased association with the lipid rafts, and subsequent palmitoylation of Cys 95 resulted in enhanced GAP activity (Osterhout JL, 2003). In this case, the RGS4 protein could negatively regulate (attenuate) mu receptor signalling at the effector rather than the receptor.

There are hundreds of G protein-coupled receptors and only approximately 30 RGS proteins. If RGS proteins do not directly interact with their receptors, then how could specificity for receptor regulation be explained? One possibility involves the distribution of RGS protein family members within cell particular populations. For example, RGS4 is found in many areas of the brain, including those with high populations of mu opioid receptors. Chronic agonist treatment results in an increasing pool of activated GTP-bound G□ subunits as well as G□□subunits, activating kinases and other downstream effectors (Nestler, 1993, 1997). One of these signals may be sufficient, either alone or in concert with others, to induce translocation of RGS4 from the nucleus to the cytosol, where it interacts with mu receptor-associated G proteins or mu receptor associated effectors, i.e. adenylyl cyclase.

Future directions

Despite recent advances in understanding the molecular adaptations to chronic opioid administration and the molecular mechanisms of actions of RGS proteins, there is no unifying hypothesis to explain the mu opioid receptor-mediated development of tolerance and dependence. Future studies will include the development of antibodies that recognize endogenous RGS4 as well as antibodies to either the mu opioid receptor or RGS4, raised in an animal other than the rabbit, that immunohistochemically identifies its specific protein. These tools will allow for quantification of changes in RGS4 protein levels in response to agonist treatment and for the co-localization of the two proteins within the same neurons. Additionally, an antibody that recognizes endogenous RGS4 would be extremely valuable to immunoprecipitate RGS4 and any proteins that associate with it.

Other studies include determining if chronic opioid treatment can induce translocation of RGS4 from the nucleus in PC12 cells (or another cell line that expresses the mu opioid receptor). If this study proves to be unattainable, then another approach would be to transfect a cell line that expresses endogenous mu receptors with RGS4 in an attempt to induce translocation with mu agonists. If induction of translocation is successful, then the mechanism of translocation would be investigated. The leucine-rich nuclear export sequence, **L**AG**L**PASC**L**, is identical to the CRM1 mediated nuclear translocation motif. Leptomycin, an inhibitor of CRM1 activity, could be tested for its ability to inhibit translocation. If chronic agonist stimulation results in translocation, then the effect of chronic

agonist treatment and inhibition of translocation on cyclase activity should be investigated. Further, activation of the mu opioid receptor activates many kinase pathways including Mitogen Activated Protein Kinase (MAPK), G Protein-Coupled Receptor Kinases (GRKs), and Protein Kinase A (PKA). Inhibitors of these kinases would be used to evaluate their effect on adenylyl cyclase activity and translocation from the nucleus.

The complexity of the development of mu receptor-mediated tolerance and desensitization can be largely attributed to the ability of opioid receptors to preferentially activate multiple signalling pathways. Though there may be several cellular mechanisms underlying this process, RGS proteins are likely to play a predominant role. As negative regulators of receptor signalling pathways, RGS proteins serve as a cellular molecular clock, rapidly resetting effector systems regulated by inhibitory G proteins. As such, further characterizing the distribution and molecular mechanisms of RGS proteins may be fundamental to developing a unifying hypothesis to characterize the development of tolerance and provide therapeutic treatments that are less likely to induce desensitization and dependence.

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Chapter 5

Addendum

Original specific aims which yielded negative results

Rhodamine-tagging of mu receptor antibody 1414

Immunohistochemical staining and *in situ* hybridization studies revealed that the mu opioid receptor and RGS4 are co-expressed in many regions of the rat brain that are associated with desensitization and dependence, including dorsal horn, caudate-putamen, striatal patches, thalamus, periaquaductal grey, hippocampus, habenula and ventral tegmental area (Mansour et al., 1995; Gold et al., 1997) and this lab (Chapter 3). The regulatory action of RGS4 in cells is dependent on its localization at or near the plasma membrane where it can contact the receptor, G proteins, and effectors. Both antibodies, against RGS4 and the mu opioid receptor, were raised in rabbits, making co-localization difficult. To determine if RGS4 protein is expressed in the same neurons that express mu opioid receptors, the mu opioid receptor antibody 1414 (raised against the C-terminal 50 amino acids of the rat mu opioid receptor) was directly tagged with rhodamine. Directly tagging the mu receptor antibodies with a fluorescent secondary prior to staining would eliminate the need to stain the sections first with one primary, then the anti-rabbit secondary, followed by the second primary and then another anti-rabbit secondary.

Methods

Coupling of rhodamine to mu receptor antibodies

Tetramethylrhodamine isothiocyanate (TRITC) was coupled to the affinity-purified antibody 1414 according to manufacturer's directions. Briefly, affinity-purified antibody 1414 (1 mg) was dialyzed overnight against 100 mM carbonate buffer (pH 9.0). Following dialysis, the volume was reduced to 0.5 ml using a Centriprep 10 Centrifugal Filter (Millipore; Bedford, MA), obtaining a final concentration of 1 mg/0.5 ml.

A total volume of 25 µl tetramethylrhodamine isothiocyanate (TRITC) dissolved in anhydrous DMSO (1 mg/1 ml) was added to the purified antibody preparation and the reaction mixture was incubated in the dark at 4ºC for 1 h. Following dark incubation, 50 µl of 500 mM ammonium chloride (final concentration = 50 mM) was added and the solution incubated at room temperature for 2 h. Finally, 50 µl 1% xylene cyanol (final concentration = 0.1%) and 50 µl of 50% glycerol were added and the unbound dye was separated from the conjugate by gel filtration using a Sephadex G50 column (Amersham Pharmacia).

For rhodamine coupling, the ratio of rhodamine to protein can be estimated by measuring the absorbance at 550 nm and 280 nm. The ratio of absorbance for rhodamine/protein (550 nm/280 nm), should be between 0.3 and 0.7.

Absorbance readings at 550nm and 280 nm

Wavelength (nm)	Absorbance
550.0 nm	0.1785 A
280.0 nm	0.5288 A

The ration of 280nm:550nm was 0.337, indicating an acceptable ratio of antibody tagging. The O.D. at 280 was 0.5288 A. Given that an $O.D._{280}$ of 1.0 represents approximately 0.75 mg/ml antibody, we had approximately 0.4 mg protein in our 1.5 ml preparation.

Immunohistochemical staining of rat brain slices with rhodamine-tagged mu receptor antibodies

Perfused rat brain sections were incubated for 2 days at 4ºC in a solution of 0.3% Triton-X 100 in PBS and 1:1000 dilution of rhodamine-tagged antibody. As a control for the primary antibody, sections were incubated in parallel with 1:1000 rhodamine-tagged 1414 that had been presorbed overnight with 10^{-7} M of antigen (20 µl GST-$MOR_{349-398}$). After incubation, sections were washed in 0.2% Triton X-100/PBS and mounted in glycerol.

Results

The rhodamine tagged anti mu receptor antibodies did not specifically label any proteins when viewed with a fluorescent microscope (580 nm) (Figure 1).

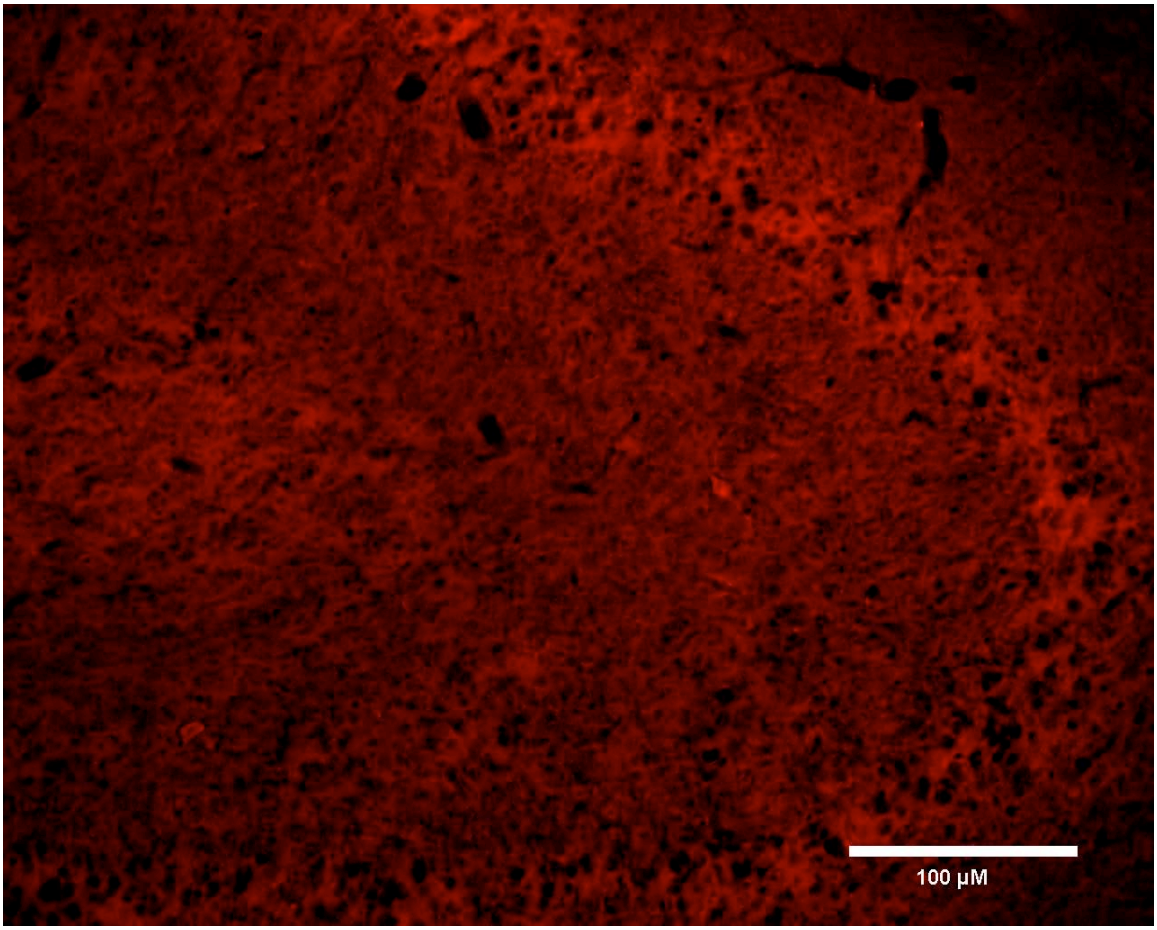

Figure 1. Representative rat brain section incubated with mu opioid receptor antibodies (1414) directly tagged with rhodamine (1:833). There is no labelling in any region.

Additional modifications and results

The initial antibody dilution of 1:1000 was chosen as a starting point because the untagged, affinity-purified 1414 worked well in immunohistochemical studies at that dilution. Because it was possible that the dilution was too weak for the directly-tagged antibody, sections were incubated as described above using 1:200, 1:500, and 1:833 dilutions of Rhodamine-tagged 1414. This approach was unsuccessful. No labelling was present in any region of the rat brain sections.

Since 7 days had elapsed from the initial absorbance reading, the ratio of rhodamine to protein was again estimated by calculation the ratio of the absorbance at 550 nm and 280 nm and was found to be 0.3203 (within acceptable range).

Wavelength (nm)	absorbance
550.0 nm	0.1647 A
280.0 nm	0.5142 A

Sections were pre-incubated for 24 h at 4ºC in 0.3% Triton-X 100/PBS, followed by addition of antibody (1:833) and incubation at room temperature for 6 h. It was hoped that the pre-incubation with detergent and room temperature incubation would allow for greater, more rapid penetration of the tissue by the antibody. This procedure was also unsuccessful. No labelling was present in any region of the rat brain sections.

Measure and compare basal and mu receptor stimulated GTPase activity in the presence or absence of recombinant RGS4

Because the primary action of RGS4 is to increase the GTPase activity of the G□ subunit, in this experiment we tried to measure the steady state GTPase activity (GAP) in SH-SY5Y cell homogenates in the presence and absence of recombinant RGS4. The assay utilizes □[^{32}P]GTP to measure the increase in the rate of GTP hydrolysis by the □ subunit of G proteins.

Methods

Recombinant RGS4 was tested for its ability to affect DAMGO-stimulated GTPase activity in SH-SY5Y cell membranes. SH-SY5Y cells were pretreated for 2 h with 1μM DAMGO, and then were incubated in fresh media (without DAMGO) for 1/2 h. Cells were centrifuged at 500 x g for 5 min, then were homogenized in buffer A containing 20 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 5.0 MgCl, 30 mM NaCl. Cellular debris was pelleted by slow centrifugation (500 x g at). Membranes were isolated by centrifugation at 48,000 x g for 20 min. at 4ºC. The resulting pellet was resuspended in 0.01 volume of Solution A and rehomogenized. To measure steady state hydrolysis of □[^{32}P]GTP by SH-SY5Y membranes in the absence or presence of purified RGS4 (3 μM), reaction mixtures containing the following components were incubated at 30ºC for 10 min.: 20 mM Tris·HCl, 0.1 mM EDTA, 5 mM MgCl, 30 mM NaCl, 1.0 mM ATP, 1.0 mM App(NHp), 1.0 mM DTT, 1.0 mM GTP, 100,000 CPM □[^{32}P]GTP, 5 U creatine phosphate, 25 mM creatine phospho-kinase, and membranes (~2 - 4.5 μg/assay).

To obtain the above solution, three stock solutions were prepared. The final concentrations for use in the GTPase assay were: **Solution A:** 20mM Tris·HCL (pH 7.4). 0.1 mM EDTA, 5.0 MgCl, 30 mM NaCl. **Solution B:** 1.0 µM ATP, 1.0 µM App(NHp). **Solution C:** 1.0 mM DTT, 1.0 mM GTP, 100,000 cpm [^{32}P]GTP, 10 mM creatine phosphate, 5 U creatine phospho-kinase.

To measure GTPase activity, reactions were prepared in triplicate on ice, as follows:

	DAMGO	**soln A**	**soln B**	**soln C**	**H_20**	**cold GTP**	**RGS4**	**protein -**
RGS4								
1-3	-	20 µl	10 µl	10 µl	50 µl	-	-	10 µl
4-6	10µl	20 µl	10 µl	10 µl	40 µl	-	-	10 µl
7-9	-	20 µl	10 µl	10 µl	40 µl	10 µl	-	10 µl
								+RGS4
10-12	-	20 µl	10 µl	10 µl	40 µl	-	10 µl	10 µl
13-15	10µl	20 µl	10 µl	10 µl	30 µl	-	10 µl	10 µl
16-18	-	20 µl	10 µl	10 µl	30 µl	10 µl	10 µl	10 µl
19	-	20 µl	10 µl	10 µl	60 µl	-	-	-

The assay was stopped by the addition of 1900 µl ice-cold 5% (w/v) Norit charcoal in 0.05 M NaH_2PO_4. All [^{32}P]GTP, but not organic ^{32}P is absorbed by the charcoal. Reaction tubes were centrifuged at 15,000 x g for 10 m at . One ml of material was removed from each tube and placed in a scintillation vial. 9.0 ml scintillation fluid was added to each vial and then counted on a Beckman liquid scintillation counter (Fullerton, CA). To determine the nonspecific membrane GTPase signal, 1 mM unlabeled GTP was added to a vial containing

only the above assay mix. This value was subtracted from the total counts per minute in the other assay tubes.

Results

There was no measurable mu receptor-stimulated GTPase activity in the assay tubes. The experiment was repeated as described above with the following modification. Prior to adding membranes all assay components were added to reaction tubes and centrifuged at 4ºC for 3 min at 1500 x g. Reactions were started by the addition of membranes to tubes at 30 s intervals and the immediate placement of tubes into a 30ºC water bath. Reactions were terminated at 30 s intervals. The mu receptor-stimulated GTPase values were extremely low (equal to background) and did not differ among any of the treatment groups.

Additional modifications and results

The additional time required to isolate the membranes from the homogenate may have contributed to the lowered GTPase activity. Therefore, the experiment was repeated with no attempt to recover membranes. Instead the original whole homogenate was used in the assay tubes. This procedure was unsuccessful, because the mu receptor-stimulated GTPase values were equal to background.

Another possibility was that there were too few mu opioid receptors (and therefore, extremely low levels of mu receptor-stimulated GTPase activity, even upon agonist stimulation) in the SH-SY5Y cell line. If this were the case, the

changes in GTPase activity might be too difficult to accurately measure within this system. The assay was repeated, but solubilized mu receptors from rat brain membranes were used. DAMGO (1 µM) and morphine (10 µM) were used as the mu agonists and the protein (containing solubilized mu receptors) was diluted sufficiently so that the volume would be 30%, rather than 10% of the total in each assay tube. The assays were repeated using 2 minute, 5 minute, 10 minute, and 15 minute incubations at 30ºC. All of these manipulations were unsuccessful. There was no detectable mu receptor stimulated GTPase activity.

Next, PC12 cells, stably transfected with the mu opioid receptor, were used in the GTPase assay. It was hoped that the numbers of mu opioid receptors would be high enough to detect mu receptor-stimulated GTPase activity in the various treatment groups, however we were unable to measure mu receptor stimulated GTPase activity.

The GTPase assay was tried 12 times. In addition to the above modifications, the percentage of total volume of RGS4 was varied and the tubes were kept in an ethanol ice bath prior to starting the reaction and immediately upon termination of reaction. None of the manipulations resulted in statistically significant differences in basal and mu agonist-stimulated GTPase activity either in the absence or presence of RGS4. The data generated from two of the experiments yielded discernable differences in GTPase activity, but the numbers were not significant (Figure 2).

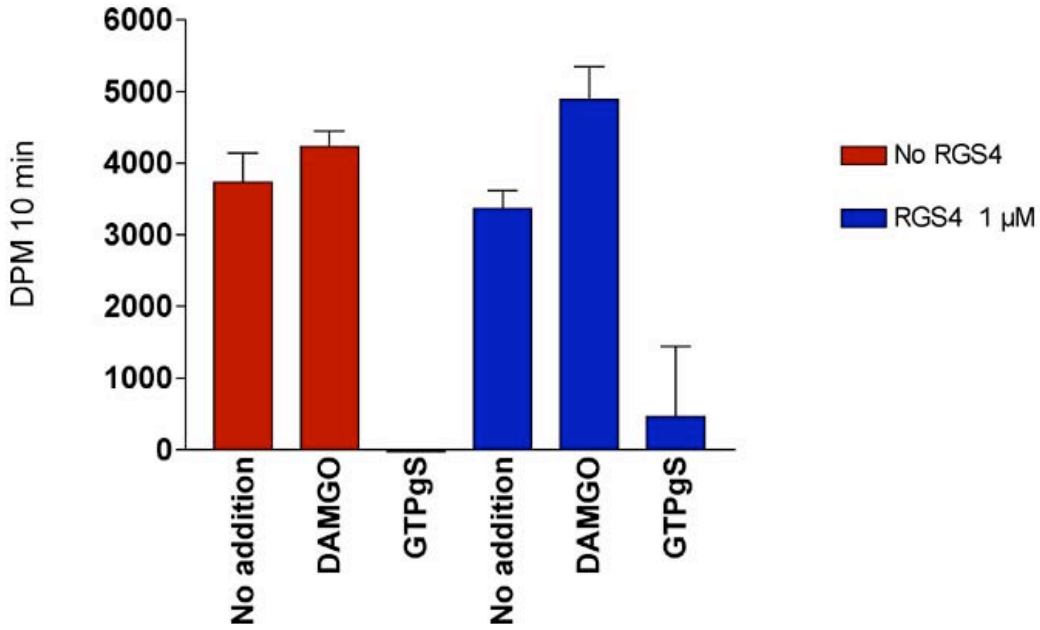

Figure 2. GTPase activity in SH-SY5Y cell membranes. There is no significant difference in hydrolysis activity in the control or DAMGO-stimulated groups ($p = 0.1468$, not significant and $p = 0.4056$, not significant, respectively.).

Treatment of PC12 cells with DAMGO, Morphine, ANP, and 8-bromo-cGMP to induce translocation of RGS4 from nucleus to cytosol

Our studies have revealed that RGS4 is primarily in the nucleus of neurons. Though other laboratories have shown RGS4 to be present in the cytosol, their studies use cell lines that have been transfected with RGS4 fusion proteins. Quite possibly, the unnaturally high levels of protein associated with transfection and/or the presence of the fusion protein tag account for the discrepancy in location of the RGS4 protein. Pedram *et al.* demonstrated that the addition of atrial natriuretic peptide (ANP) to a PC12 cell line containing stably transfected mu receptors resulted in a translocation of a 30 kDa protein that was immuno-precipitated with RGS4 antibodies from the cytosol to the membrane (Pedram et al., 2000). This effect was blocked by the addition of an inhibitor of guanylate cyclase activation or by an inhibitor of cGMP-dependent protein kinase (PKG). In these studies, ANP phosphorylated the 30 kDa protein (via PKG), and the inhibition of PKG blocked translocation. The translocation was likely a result of RGS4 phosphorylation by atrial natriuretic peptide-mediated activation of PKG.

We hoped to determine if atrial natriuretic peptide and 8-bromo-cGMP could induce endogenous RGS4 translocation in these same PC12 cells. Additionally, because chronic treatment with two mu specific agonists, DAMGO and morphine (Nakagawa et al., 2001), increased RGS4 mRNA levels in PC12 cells transfected with mu receptors, we also attempted to determine if DAMGO or morphine could induce RGS4 translocation from the nucleus to the cytosol.

Methods

A rat pheochromocytoma cell line (PC12 cells), stably transfected with the mu opioid receptor (a kind gift of M. Satoh), were grown to ~80% confluency in Lab-Tek II Chamber Slides (VWR Scientific Products) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% horse serum, 5% fetal calf serum, 50U/ml penicillin, 50 ug/ml streptomycin, 200 µg/ml G418. Prior to the addition of drugs the media was changed (0.3 ml/well). Cells were treated with no drug or with DAMGO (1 µM for 2 h), morphine (10 µM for 2 h), ANP (10 nM for 2 min), or 8-Br-cGMP (1 µM for 2 min). Following appropriate incubation times, the media was removed and the wells were washed with PBS prior to fixing in 4% paraformaldehyde. Fixed slides were subject to immunohistochemical staining with affinity-purified anti-rat RGS4 antibodies.

Results

There were no consistent differences in sub-cellular location of RGS4 between the control and treated groups. RGS4 was primarily localized in the nuclei of all cells in all treatment groups.

Immunoprecipitation of mu opioid receptors from rat brain membranes incubated with recombinant RGS4

To determine if RGS4 forms a physical association with the mu receptor and/or its cognate G proteins as a result of chronic mu receptor activation, we incubated recombinant RGS4 with brain membranes from rats that had been chronically treated with fentanyl. Mu receptors were solubilized and immunoprecipitated with affinity purified mu receptor antibodies directed against GST fusion proteins raised against the C-terminal 50 amino acids (1414) and 61 amino acids from the N-terminal region (1404) of the mu receptor as described previously (Chalecka-Franaszek et al., 2000). The immunoprecipitated pellets were screened in a western blot with affinity purified antibody raised against the C-terminal 36 amino acids of RGS4 (1554).

We anticipated the potential physical association of RGS4 with the C-terminal residues of the mu receptor. Tethering of RGS4 to the C-terminus would place the protein in close proximity to the receptor's cognate G proteins. If our hypothesis was correct, then the material immunoprecipitated with the C-terminal antibodies would not contain RGS4, while the material immunoprecipitated with the N-terminal antibodies would contain RGS4.

Methods

Solubilization and Immunoprecipitation of Mu opioid receptors from rat brain membranes.

Male, Sprague-Dawley rats (~150 g) were treated with 0.056 mg/kg fentanyl. After 2 hours the rats were decapitated and active mu opioid receptors

were solubilized from membranes as previously described, with the following modifications (Chalecka-Franaszek et al., 2000). Receptors were solubilized in buffer containing 50mM Tris, 100 mM NaCl, 2 mM Tris·EDTA, 1 mM Tris·EGTA, 5mM $MgCl_2$, 0.1 mM PMSF, 1 µM leupeptin, 1 µM pepstatin, 2 µg/ml aprotinin, 5 µg/ml trypsin inhibitor, and 100 µM $NaVO_4$ (Buffer T). Following centrifugation (48,000 x g [22,000 rpm]), membranes were applied to Sephadex G50 columns pre-equilibrated with Buffer T containing 4 mM Chaps. Material was precleared by incubating with Prot A Seph CL4B at 4ºC for 30 min. Precleared material was divided into six groups and immunoprecipitated overnight at 4ºC with specific mu receptor antibodies as follows:

Group	Immunoprecipitation material
1	-specific mu receptor antibody 1414 (raised against GST-C50, a fusion protein consisting of GST and the C terminal 50 amino acids of the mu opioid receptor) -10^{-7} M GST
2	-specific mu receptor antibody 1414 -10^{-7} M GST-C50
3	-specific mu receptor antibody 1414 -10^{-7} M GST -10^{-3} M GTPγS
4	-specific mu receptor antibody 1404 (raised against GST-N61, a fusion protein consisting of GST and 61 amino acids from the N terminus of the mu opioid receptor) -10^{-7} M GST
5	-specific mu receptor antibody 1404 -10^{-7} M GST-N61
6	-specific mu receptor antibody 1404 -10^{-7}GST -10^{-3} M GTPγS

Both mu receptor antibodies were affinity-purified and crosslinked to Protein A. Sepharose. Following overnight incubation at 4ºC with constant rotation, tubes were microfuged for 20 sec, and pellets containing solubilized receptors were washed 5X in Buffer T with 4 mM Chaps. (Pellets from material that had been incubated with antibody and GTγS were washed in buffer T with 4 mM Chaps and 1 µM GTPγS.) Washed pellets were heated for 5 min. at 100ºC in 1x SDS sample buffer without dithiothreitol (DTT) to minimize the release of heavy and light chain of IgG from the resin. The resin was removed by centrifugation, and DTT was added to the supernatants; the material was then incubated for 1 h at 37ºC.

Western blot detection of RGS4 and mu opioid receptors from immunoprecipitated material

Resulting samples were subject to SDS-PAGE on a 12% gel for 4 h at 180V. Following SDS-PAGE, the proteins were transferred to nitrocellulose membrane (Millipore, Immobilon-P transfer membrane). The blots were incubated overnight at 4ºC in a solution of 5% nonfat milk in phosphate buffered saline containing 0.10% Tween 20 (TBST) and were then subjected to Western blot analysis using RGS4 antibodies (1554) generated against a fusion protein of GST and the C-terminal 36 amino acids of rat RGS4 or mu opioid receptor antibodies (1414) generated against a fusion protein of GST and the C-terminal 50 amino acids of the rat mu receptor. To detect RGS4, membranes were incubated for 1 h at room temperature with 0.5 µg/ml affinity purified antibody 1554 alone or presorbed with 10^{-7} M GST-C36. To detect mu opioid receptors,

membranes were incubated for 1 h at room temperature with 5.0 µg/ml affinity purified antibody 1414 alone or presorbed with 10^{-7} M GST-C50. Membranes were washed in TBST, incubated in donkey anti-rabbit horseradish peroxidase (1:50,000; Pierce) for 1 h at room temperature, then were washed with TBST. Peroxidase labelled antibodies were detected with ECL western blotting detection reagents (Pierce; West Pico, Super Signal Chemiluminescent Substrate) and exposure to Hyperfilm-ECL (Amersham). Films were developed in an automatic film processor (Kodac M35A X-Omat processor).

Results

Western blot analysis of immunoprecipitation products, using anti-RGS4 antibodies, did not demonstrate the presence of a band of the correct size (~26,000 kDa) that was blocked (Fig. 3). We were not able to show a physical interaction of RGS4 with the mu opioid receptor under these conditions.

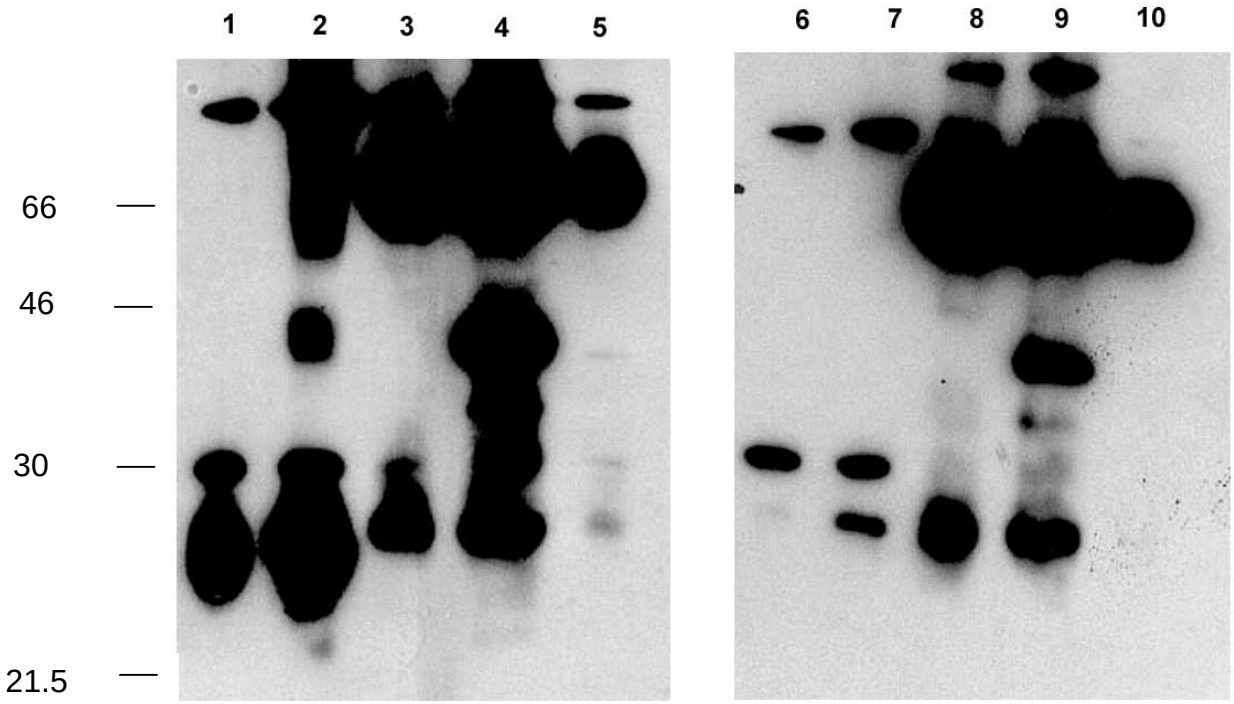

Figure. 3 Screening for recombinant RGS4 in material co-immunoprecipitated with mu opioid receptor antibodies. The pelleted material, containing solubilized mu opioid receptors, does not demonstrate the presence of a band the correct size (~26,000 kDa) that was blocked by the addition of 10^{-7} M antigen. Left panel represents material screened with 0.5µM anti-RGS4. Right panel represents material screened with 0.5µM anti-RGS4 that had been presorbed with 10^{-7}M antigen. Lanes 1, 2, 6,and 7: recombinant RGS4 runs as a band ~26,000 kDa. Lanes 3 and 8: Material immunoprecipitated with 1404. Lanes 4 and 9: Material from blocked immunoprecipitation blocked with N 61. Lanes 5 and 10: Material immunoprecipitated with 1414.

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